Development of Antisense H-Ras Oligomers and Investigation of Their Potential as a New Drug Against Liver Cancer: In Vitro and In Vivo Studies

Thesis submitted

By

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"Statement of Originality"

I, **Alankar Mukherjee**, registered on **10th May, 2022**, do hereby declare that this thesis entitled **" Development of Antisense H-Ras Oligomers and Investigation of Their Potential as a New Drug Against Liver Cancer: *In Vitro* and *In Vivo* Studies"** contains literature survey and original research work done by the undersigned candidate as part of Doctoral studies. All information in this thesis have been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that, as required by these rules and conduct, I have fully cited and referred all materials and results that are not original to this work. I also declare that I have checked this thesis as per the "policy on Anti Plagiarism, Jadavpur University, 2019", and the level of similarity as checked by iThenticate software is less than 6%.

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled " **Development of Antisense H-Ras Oligomers and Investigation of Their Potential as a New Drug Against Liver Cancer: *In Vitro* and *In Vivo* Studies** " submitted by **Mrs. Alankar Mukherjee, (Registration No. 1022213004 Index No. 05/22/PH)** who got her name registered on **10th May, 2022**, for the award of Ph.D. (Pharmacy) degree of Jadavpur University is absolutely based upon his own work under the supervision of **Dr. Tapan Kumar Giri** and that neither her thesis nor any part of it has been submitted for any degree/diploma or any other academic award anywhere before.

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Place: Jadavpur, Kolkata

"I dedicate this thesis to My Parents & Family for their constant support and unconditional love…"

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Preface

The present research, undertaken for the fulfillment of the requirements for the Doctor of Philosophy (Pharmacy) degree, was conducted under the esteemed supervision of Prof. (Dr.) Tapan Kumar Giri Department of Pharmaceutical Technology, Faculty of Engineering and Technology, Jadavpur University, Kolkata, India.

According to the World Cancer Report 2020, cancer was one of the leading causes of death worldwide, with an estimated 10 million deaths in 2020. The National Cancer Institute (USA) reports that cancer exhibits a sex-based disparity, with a mortality rate of 189.5 per 100,000 males compared to 135.7 per 100,000 females before the age of 70. In most countries, it remains the primary cause of mortality (WHO, 2019). Hepatocellular carcinoma (HCC), characterized by a high mortality rate, is typically diagnosed at an advanced stage where chemotherapy remains the primary treatment. However, conventional chemotherapeutic agents are often highly cytotoxic and yield limited therapeutic success.

Recent advances in gene therapy, particularly using antisense oligonucleotides (ASOs), have demonstrated promising results in inhibiting HCC without the significant adverse effects seen with traditional chemotherapy. This study aimed to evaluate the therapeutic potential of phosphorothioate-modified ASOs (PS-ASOs) targeting the mutant **H-Ras** gene, in comparison to a commercial docetaxel formulation (Taxotere®), in both in vitro and in vivo models of HCC.

Initial experiments assessed the efficacy of ASOs in suppressing mutant H-Ras expression in chemically induced HCC in rats. Further investigations targeted **H-Ras** and **c-raf.1** gene mutations—known contributors to HCC progression via the Ras-Raf-MAPK pathway. A comprehensive set of pharmacokinetic, biochemical, histological, histochemical, and morphological analyses, including confocal and electron microscopy, supported the evaluation of therapeutic outcomes.

The study include; Physicochemical characterization of ASOs, In vitro hemolysis, pharmacokinetics, hepatic uptake, and biodistribution, Antineoplastic efficacy in carcinogen-induced HCC in rats, Immunohistochemical analysis of markers such as Hep-Par I, CK-7, CD-15, and p53, In situ hybridization for gene expression analysis, Caspase-3/9 activation studies to assess apoptosis, PS-ASO demonstrated minimal hemolytic activity (<3%), time-dependent liver uptake, and extended systemic circulation. It was well-tolerated in healthy rats and showed improved therapeutic outcomes compared to docetaxel. After 6 weeks, PS-ASO treatment significantly reduced tumor incidence, suppressed H-Ras expression, increased p53 levels, activated caspase-mediated apoptosis, and prevented lung metastases. It exhibited a better safety profile and superior efficacy in reversing hepatic marker enzyme alterations than DXT.

In vitro, PS-ASOs induced apoptosis and mitochondrial membrane depolarization in HepG2 and Huh7 human HCC cells, while sparing normal liver cells. IC_{50} values indicated selective cytotoxicity. ASOs also reduced liver lesion counts and tumor burden in vivo. Notably, ASO targeting mutant H-Ras was more effective than ASO against c-raf.1, underscoring the therapeutic relevance of H-Ras inhibition in HCC management.

In conclusion, PS-ASO therapy targeting the mutant H-Ras gene presents a potent, selective, and safer alternative to conventional chemotherapy for HCC. This strategy holds promise not only for HCC treatment but potentially for other Ras-mutated malignancies. Further investigation and clinical translation are warranted.

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ABBREVIATIONS

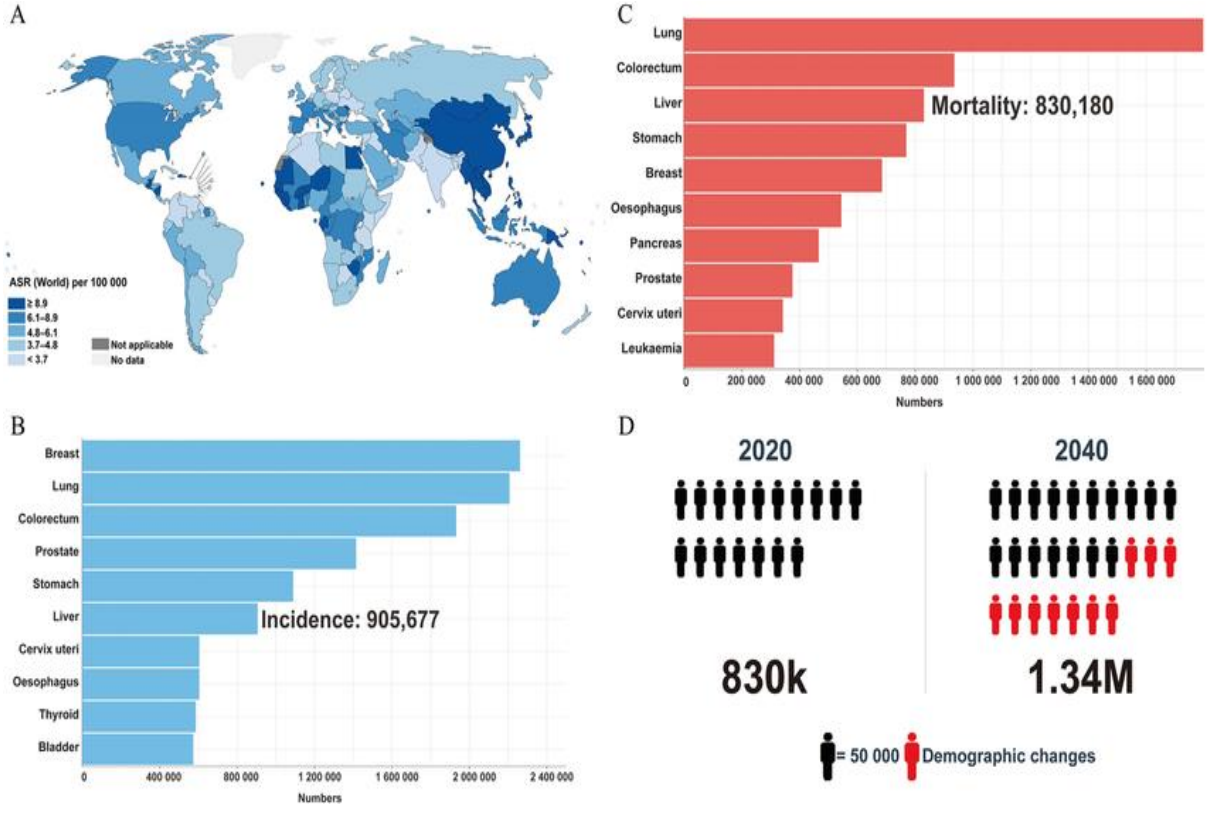
ABBREVIATIONS	DEFINATION
PS-ASO	Phosphorothioate antisense oligonucleotide
HCC	Hepatocellular carcinoma
H-Ras	Harvey-Rat sarcoma
FAM	Fluorescein Amidite
AAF	Acetyl amino fluorine
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco‟s modified eagle medium
DMSO	Dimethylsulfoxide
DLS	Dynamic light scattering
DTX	Docetaxel
eV	Electron volt
FITC	Fluorescein isothiocyanate
FBS	Fetal bovine serum
SEM	Scanning electron microscope
FTIR	Fourier-transform infrared spectroscopy
h	Hour
PBS	Phosphate-buffered saline
TEM	Transmission electron microscopy
PDI	Polydispersity index
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay
AFM	Atomic Force Microscope
SGOT	Serum glutamic-oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
ALP	Alkaline phosphatase

Chapter 1

Introduction

Introduction

One of the most challenging medical disorders to treat therapeutically is hepatocellular carcinoma (HCC), maximally prevalent primary hepatic cancer. HCC is responsible for producing the fourth-most cancer-related deaths globally and the sixth most aggressive type of cancer (Devarbhavi et al. 2023, Llovet et al. 2021). According to WHO estimates (Fig.1.1), by 2040, the annual number of new (diagnosed) cases and deaths from liver cancer may surpass 1.4 million and 1.3 million, respectively (WHO 2022).



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People with underlying chronic liver diseases often develop HCC, a very aggressive cancer that starts in the hepatocytes. HCC usually develops in people with chronic liver disease, especially those who have cirrhosis brought on by hepatitis B or C infections, alcoholism, or non-alcoholic fatty liver disease (Mukherjee et al. 2023). Heavy drinking, obesity, NAFLD (non-alcoholic fatty liver disease) that often leads to cirrhosis, a crucial prelude to liver cancer. Persistent viral infections such as hepatitis B and C are risk factors for HCC. Inherited metabolic diseases including hemochromatosis and exposure to aflatoxins, a toxin made by some molds, could be additional contributing causes.

With symptoms like ascites, jaundice, stomach pain, and weight loss, it frequently manifests in its later stages. A biopsy is frequently used in conjunction with imaging methods such as ultrasound, CT scans, or MRIs to confirm the diagnosis. The diagnosis may also be supported by increased blood levels of alpha-fetoprotein (AFP), however, this indicator is not always accurate. In certain situations, hepatic tissue biopsy may be necessary for a conclusive diagnosis.

The fact that many patients still have poor prognoses emphasizes how crucial early detection and care are. Early identification of HCC is difficult since it frequently exhibits no symptoms in its early stages. Other than the above-mentioned symptoms such as weariness, unexpected weight loss, jaundice, upper abdominal pain, and ascites swelling intensify, a number of patients may also experience less frequent but potentially concerning paraneoplastic symptoms such hypoglycemia or hypercalcemia (Mukherjee et al. 2023). The development of HCC has a long latent period and is largely asymptomatic in its early stages (Mukherjee et al. 2022). Because of this, the disease is typically discovered at an advanced stage (Mukherjee et al. 2022), making chemotherapy the sole therapeutic choice. Multi-kinase inhibitors and immunomodulators are two examples of chemotherapies that were introduced with high expectations for improving HCC patient survival (Ahn and Ursini-Siegel 2021). But the clinical results fell well short of expectations. Cytotoxic medications make up the majority of anticancer medications. Chemotherapeutic resistance and the advancement of HCC are caused by their poor target capability, low solubility, and insufficient neoplastic cellular and tissue internalization (Mukherjee et al. 2023). These characteristics significantly limit the cancer treatment efficacy and become a growing obstacle to it (Mukherjee et al. 2023). Furthermore, typical chemotherapeutic combinations have little effect on patient survival (Mukherjee et al.

2022), although the chemotherapy remains the primary treatment for HCC. Fig. 1.2 shows the various stages of liver cancer.

Trans-arterial chemoembolization, systemic treatments, or immunotherapy may be necessary for advanced cases of HCC, whereas surgical resection or liver transplantation may be used for early-stage cases, which is often rare (Mukherjee et al. 2023). HCC, in contrast to many other malignancies, is typically diagnosed at an advanced stage, at which point chemotherapy is the only treatment available, excluding radiation and surgery (Mukherjee et al. 2023). The course of treatment is determined by the disease's stage, the liver functioning state, and the patient's general condition. Surgical options for early-stage HCC patients with cirrhosis include liver transplantation, which offers the best prognosis, or partial hepatectomy, which removes the tumor and surrounding liver tissue. For patients with more severe disease, local therapy such as trans-arterial chemoembolization (TACE) or ablation therapies (radiofrequency or microwave ablation) may be used. For individuals with advanced or metastatic HCC, systemic treatments are employed, such as immunotherapies or targeted medicines like sorafenib.

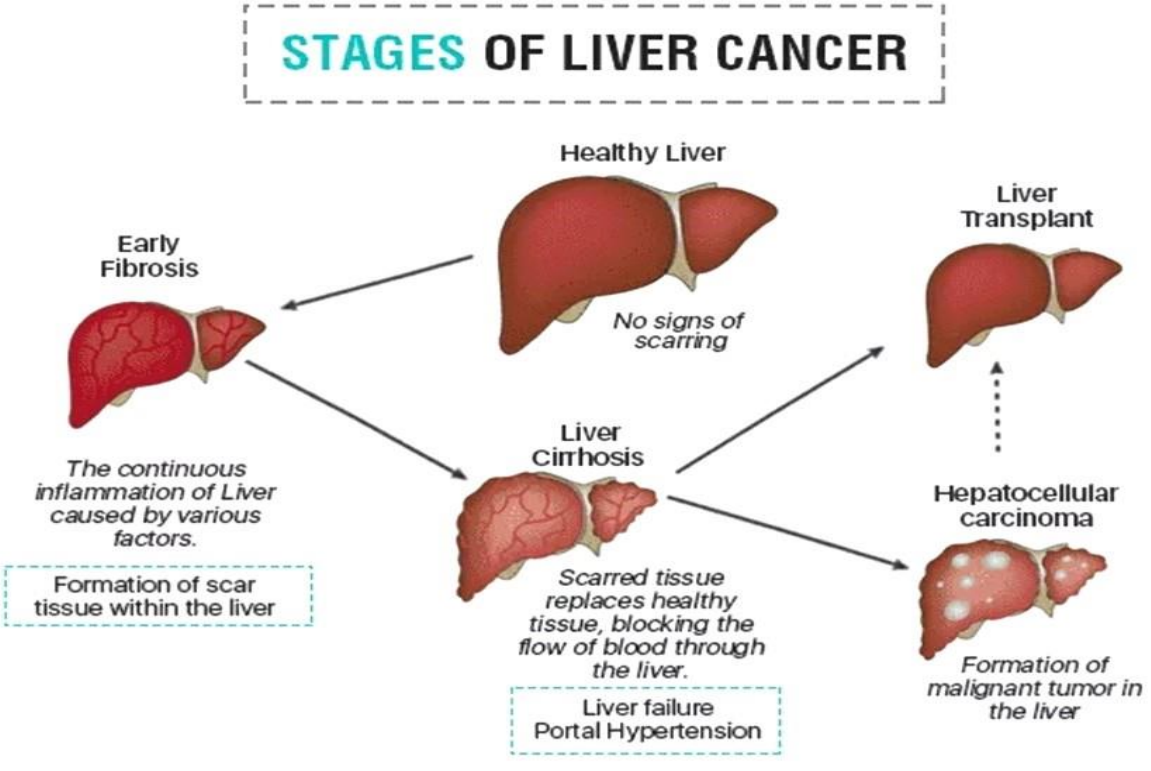

Fig. 1.2. Stages of liver cancer (Ref. https://www.ailbsindia.com/liver-cancer-symptoms-causes-treatment/)

Even with improvements in treatment, HCC still has a dismal prognosis, with a high recurrence rate following therapeutic measures. In order to identify HCC at an earlier, more curable stage, surveillance programs that keep an eye on high-risk groups such as individuals with cirrhosis or chronic hepatitis are essential. In an effort to improve outcomes for patients with this difficult malignancy, researchers are still investigating new therapies and strategies, like as gene therapy and immunotherapy. Cellular gene blocking has frequently been demonstrated to have outstanding therapeutic efficacy against different malignancies (Lu et al. 2022; Khazak et al. 2007). In contrast to the other chemotherapeutic anticancer drugs, genetic therapies, such as antisense oligomers, demonstrated strong HCC inhibitory potentials with negligible or no toxicity (Lu et al. 2022; Mukherjee et al. 2022).

Antisense oligonucleotides (ASOs) are a type of genetic therapy that targets mRNA or DNA precursors to suppress oncoproteins (Rossor et al. 2018). ASOs, a very promising family of therapies based on short nucleotide chains (single-stranded), have gained attention as a possible treatment option to meet unmet medical needs. Six ASO medications have been licensed by FDA (Food and Drug Administration of the United States), in the past seven years, and many more are in the works (Shadid et al. 2021). The use of biological response modifiers in cancer treatment has grown in popularity. Through Watson-Crick base pairing, an ASO, which is usually a single-stranded sequence (of 15–25 nucleotides) that targets a particular gene sequence/messenger RNA, selectively blocking transcription/ translation of target protein(s), hence, inhibiting gene expression (Crooke et al. 2018). They frequently undergo chemical modification to increase their stability in biological fluids. (Mukherjee et al. 2005). The most popular and extensively utilized alteration that improves its stability in bodily fluids including blood is phosphorothioate (PS) in its backbone, which involves substituting sulfur for one non-bridging oxygen at the oligonucleotide backbone. Significant metabolic stability and sequence specificity were demonstrated by PS-oligomers showed metabolically stable and specific to its target, which prevented off-target effects (Shadid et al. 2021). Commercially available ASO therapeutics, clinical trials, and a number of in vitro (Mukherjee et al. 2022) and in vivo (Mukherjee et al. 2005, 2022, 2023) investigations help increase efforts and rekindle interest in ASO among researchers. ASO has emerged as a new modality for targeted treatments and precision medicine throughout the past six to seven years (Scharner and Aznerez, 2021). ASO is a more appealing option than conventional chemotherapies due to its reduced pro-

inflammatory response, enhanced nuclease resistance, and higher target capability and binding ability with notable pharmacokinetic and pharmacological properties (Scharner and Aznerez 2021).

Targeting particular gene or messenger RNA (mRNA) molecules, antisense oligomer technology is a potent molecular biology tool that can be utilized to modify gene expression. The expression of the gene product can be changed by antisense oligonucleotides, which attach to their target DNA/ mRNA and ultimately stop or change the gene translation. This technology along with others holds great promise for therapeutic applications, particularly in the treatment of genetic abnormalities and diseases characterized by abnormal gene expression.

Through their ability to hybridize with complementary DNA/ mRNA sequences, antisense oligonucleotides can silence or modify genes in many ways (Lu et al. 2022; Crooke et al. 2018). The principal mechanisms consist of:

1. Degradation by RNase H: An enzyme called RNase H identifies and cleaves the RNA strand of an RNA-DNA hybrid. The mRNA is broken down by RNase H, which stops protein synthesis when an antisense oligonucleotide binds to its complementary mRNA to produce a hybrid molecule.

2. The ribosome or other regulatory proteins may be physically blocked from accessing the mRNA by antisense oligonucleotides, which would stop translation and the synthesis of proteins. This can be particularly helpful in situations where the objective is to prevent the synthesis of a particular protein without totally breaking down the mRNA.

3. Splicing Modulation: Certain exons may be included or excluded as a result of antisense oligonucleotides' ability to attach to pre-mRNA and obstruct the splicing process. Changed protein isoforms may be produced as a result, which could be helpful in fixing genetic abnormalities or altering proteins linked to disease.

To increase their stability, specificity, and effectiveness, antisense oligonucleotides undergo chemical modification. The ability of the ASOs to efficiently target the DNA/mRNA without

being rapidly broken down or eliminated by the body depends on these changes. Typical alterations consist of:

• Phosphorothioate backbone: By adding sulfur replacing one non-bridging oxygen atom of phosphate in the strand, the oligonucleotide becomes more stable and resistant to nucleases, which are enzymes that break down DNA and RNA.

• 2'-o-Methyl/ 2'-o-methoxyethyl modifications: These changes increase the ASO's binding affinity to its target mRNA and have the potential to lower immune activation. They also strengthen the oligonucleotides' defenses against nuclease degradation.

• Peptide nucleic acids (PNA): These are artificial DNA analogs that have a peptide-like structure in place of the sugar-phosphate backbone. Although PNAs are extremely stable and have a high affinity for RNA, their absorption by cells may be a drawback.

• Locked nucleic acids (LNA): LNAs are modified nucleotides that have an increased affinity for binding RNA because the ribose ring is "locked" in a certain configuration. They are therefore very good at focusing on particular mRNA sequences.

Antisense oligomer technology has great potential for treating a number of illnesses, particularly hereditary conditions where a single gene defect is the source of the illness. The following are a few important therapeutic uses:

Gene disorders: ASOs can be employed to quiet or fix the mutant genes that cause these conditions. ASOs, for instance, can be made to bypass exons in the dystrophin gene in conditions like Duchenne muscular dystrophy (DMD), by restoring reading frame and producing functional proteins. Another instance is spinal muscular atrophy (SMA), in which gene of survival motor neuron (SMN) is expressed more when ASOs are employed.

Cancer: ASOs can be employed for restoring tumor suppressor gene expression and to silence oncogenes. To increase cancer cell death, ASOs, for example, can target mutant p53 mRNA or suppress the expression of anti-apoptotic genes like *Bcl-2*.

Neurodegenerative diseases: Antisense oligonucleotides can be made to decrease the expression of harmful proteins, like tau or mutant huntingtin, which build up and cause neurodegeneration in conditions such as Huntington's disease, amyotrophic lateral sclerosis (ALS), and Alzheimer's disease.

Viral infections: By focusing on the viral RNA, antisense oligonucleotides can also be employed to prevent the spread of viruses. Because ASOs can interfere with viral replication, this has been investigated in the treatment of diseases like hepatitis C and HIV.

Autoimmune diseases: ASOs can be employed to treat autoimmune diseases, namely, multiple sclerosis, lupus, and rheumatoid arthritis by modifying gene expressions linked to immune response. ASOs, for instance, can be made to target cytokine mRNA, which lowers inflammation and over-activity of the immune system.

Notwithstanding the enormous potential of antisense oligomer technology, several hurdles need to be conquered before it can be widely used in therapeutic settings:

Delivery: Making sure ASOs are delivered to target cells effectively is one of the biggest challenges for their application in clinical settings, particularly for illnesses that affect tissues like the brain or muscles. Cell membranes and the blood-brain barrier are two biological barriers that the delivery systems must be able to cross.

Off-target effects: Although ASOs are very specific, unexpected mRNA sequences may bind to them, which could have negative consequences. Given the possibility of many mutations in complicated disorders like cancer, this problem is especially crucial when employing ASOs.

Immunological activation: Some antisense oligonucleotides can still elicit immunological responses, which might result in inflammation or other adverse effects, even while changes are made to lessen immune activation.

Cost: Antisense oligonucleotide development and synthesis can be costly, which hinders their wider application in healthcare, particularly in low-income environments.

Clinical investigations have already shown that antisense oligomer technology works. The use of this technique reached a significant turning point with the approval of Spinraza (nusinersen), an ASO therapy for spinal muscular atrophy. The potential for ASOs to cure inherited disorders by directly addressing genetic abnormalities has been demonstrated by the approval of Eteplirsen, another ASO, for treating DMD.

A number of interesting candidates are presently undergoing clinical testing as part of the ongoing investigation into the potential use of antisense oligonucleotides treating viral infections, neoplasia, and neurological illnesses.

With the capacity to accurately blocking gene or alter gene expression at the RNA level, antisense oligomer technology is a state-of-the-art method of gene control. Even though there are still issues with off-target effects and effective distribution, the advancements in clinical applications highlight the therapeutic promise of ASOs. Antisense oligonucleotides have the potential to be a key component of personalized medicine, offering therapy options for a variety of acquired and hereditary disorders as delivery methods advance and more is known about their long-term effects.

Additionally, gene therapy is being investigated as a cancer treatment, mostly using strategies like immune system augmentation and gene editing. CAT cells are T cells when chimeric antigen receptor-introduced into the T cells. As previously identified, CAR-T cell therapy have demonstrated remarkable efficacy in treating diseases such as lymphoma and leukemia by improving the T cells' capacity to detect and combat cancer cells.

The formation and progression of liver cancer are frequently linked to the overexpression of mutant genes, including Ras, Raf, myb, myc, etc. (Sun et al. 2022; Gedlay et al. 2010; Das et al.2010; Moser and Fabbro1996). In HCC, Ras/raf/MAPK signaling pathway is frequently hyper-activated (Sun et al. 2022, Gedaly et al. 2010). Many mutations in the *H-Ras* gene cause HCC to develop, progress, and spread (Yang and Liu, 2017). It may also be "a reliable and dependable marker" for HCC diagnosis and all-encompassing HCC treatment (Yang and Liu 2017; Sui et al. 2012). It is unknown, however, how these *H-Ras* mutations start the

carcinogenesis process. A glycine to valine mutation in *H-Ras* codon 12 that subsequently activates p21 protein irreversibly in a guanosine 5'-triphosphate (GTP)-bound state, exhibiting remarkable oncogenic ability (Ahmadian et al. 1999), is widely acknowledged as a hallmark in animal and human primary liver cancer (Cullen et al. 2011).

Long linked to the onset and spread of HCC are the H-ras mutation (located in codon 61 at position 12) and the Raf-1 (c-raf.1) mutation (located at CRAFP261A) in the ras-raf-MAP kinase pathway (Fig. 1.3) (Sun et al. 2022; Gedlay et al. 2010; Das et al. 2010). ASO selectively suppresses c-raf-1 kinase gene expression and the neoplastic cell proliferation growth in cell culture and tumor growth in vivo using animal models of human tumor xenografts (Monia et al. 1999).

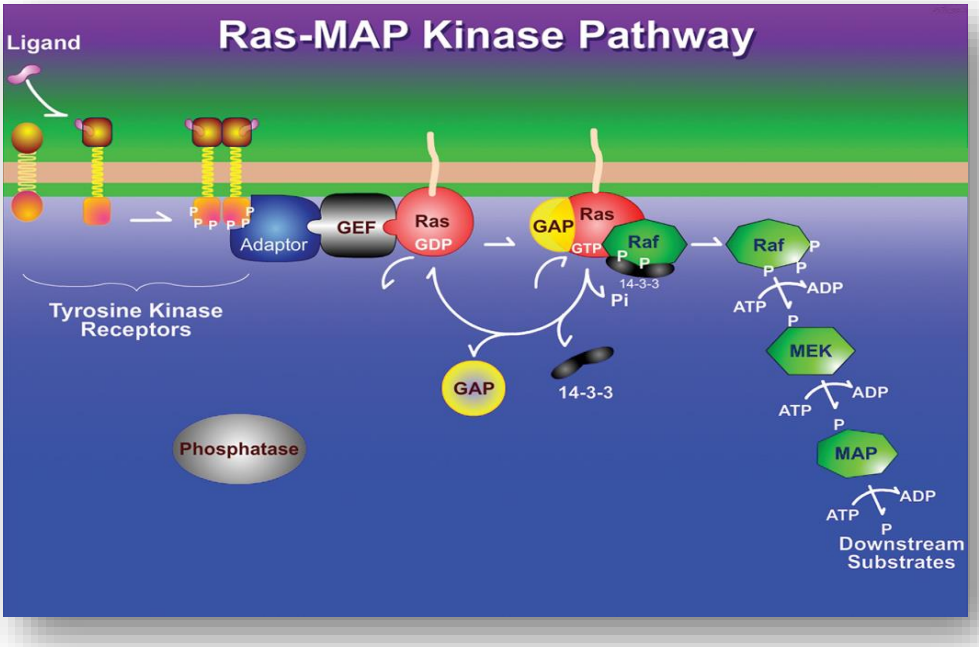

Fig. 1.3. Ras Raf Mapk Pathway
(Ref: http://stke.sciencemag.org/cgi/content/full/3/119/tr1/DC1)

In this study, we have examined the effectiveness of taxotere®, a commercially available formulation of docetaxel, and *H-Ras*-targeted gene therapy in preventing carcinogen-induced HCC in rats. The conclusion was reached by analyzing a number of biochemical, histological,

pharmacokinetic, histochemical, and morphological data in addition to confocal and electron microscopic studies.

Antisense oligomer against the mutant H-ras created by us was used and antisense oligomer sequence against c-raf.1 was selected from the reported investigations documented elsewhere (Khazak et al. 2007; Monia et al. 1999; Moser and Fabbro1996). We have chosen two antisense oligomer sequences for this investigation: one against c-raf.1 (ASO2) (Khazak et al. 2007; Moser and Fabbro1996) and one against H-ras (ASO1). In the current study, we attempted to use selective phosphorothioate backbone-modified antisense oligomers to inhibit mutant H-Ras/c-raf.1-overexpression separately in rats in order to compare their therapeutic potential to inhibit HCC in rats. To be more specific, we aimed to examine and contrast the effects of targeted gene therapy against the mutant *H-Ras/c-raf.1* gene in preventing HCC in rats for a mechanistic understanding.

Chapter 2

Review of literature

Review of literature

An overview of cancer

Cellular proliferation is essential for maintaining basic physiological functions in multicellular creatures, such as humans Cell proliferation requires for embryonic development, organ and tissue growth, normal biological activity, and wound healing. Cellular proliferation in biology refers to cell division from a parent cell into daughter cells, increasing the number of cells, and cell expansion, which builds up of cell mass and increases the cell dimension in size. Intracellular gene regulatory networks and external growth factors tightly coordinate cellular proliferation. The damaged or dead cells are always removed. Thus, a homeostasis of cell proliferation and cell death is preserved.

But when that genetic network breaks, the whole thing falls apart, leading to unchecked, aberrant cell division and frequently a lump of cells in solid tissues, like a tumor or neoplasm, or an abnormal enhancement of special cell types. Tumors are divided into two groups according to their traits and capacity to spread. The benign tumors possess some specific traits. At the cellular level, the basal membrane is still intact, and the development rate is slower than in other types of tumors and kept in and surrounding the area of origin. Since the cells remain in their primary place and are unable to move to surrounding tissue or a distant body area, they lack the ability to invade. Abnormal cell masses proliferate more quickly than their neighboring cells, fail to die, and spread at the nearby or distant tissues or organs are malignant. All carcinomas, sarcomas, adenoma, leukaemia, lymphoma, etc. are the examples of malignant cell types. The mass of cells that have the potential to develop into malignant tumors if treatment is not received constitutes another extremely small category of precancerous or premalignant tumors, for instances, dysplasia, colon polyps, and keratosis.

Cancer epidemiology: an abridged view

An estimated 10 million cancer-related deaths of humans in 2020 became one of the key causes of death worldwide (World Cancer Report 2020). The National Cancer Institute (NCI) claims that cancer deaths are sex biased and more in males than in females. Cancer-related mortality was189.5 per 100,000 male participants vs. 135.7 per 100,000 female subjects before the age

of 70, and thus, cancer-related death played a critical role in most nations (WHO, 2019). The delay or lack of a diagnosis appeared to lower the incidence rate, which was followed by a spike in illness progression and increased mortality (Englum et al. 2022). The detection and treatment of cancer were negatively impacted in 2020 by limited access to hospitals and clinics as a result of the coronavirus pandemic.

The incidence of cancer has been rising rapidly in recent years, mostly as a result of changes in lifestyle and related behaviors. According to NCI's (2017) statistical cancer survey from 2015–2017 data, 39.5% of people are predicted to receive a cancer diagnosis in their lifetime. Till the first month of 2019, approximately 16.9 million subjects were cancer survivors in the USA, and by 2030, that number is predicted to rise to roughly 22.2 million.

Various forms of radiation, including ionizing radiation from X-rays, industrial radioactive materials, and ultraviolet radiation from sunshine, are physical cancer-related risk factors (Irigaray and Belpomme 2010).

A popular food contaminant, aflatoxin, arsenic, a contaminant of drinking water, nickel, cadmium, asbestos, vinyl chloride, alcohol, tobacco smoke, benzidine, benzene, benzidine, and several air pollutants are well-known carcinogens.

Biological carcinogens (BAs) are parasites, viruses, bacteria. About 13% of cancers identified worldwide in 2018 were caused by BA-linked infections, which include *Helicobacter pylori bacteria*, hepatitis B virus (HBV), Epstein-Barr virus, hepatitis C virus (HCV) and human papillomavirus (HPV) (Irigaray and Belpomme 2010). In addition to directly causing cell damage, BAs also raise the risk of developing other cancers; for example, an HBV or HCV infection raises the risk of developing liver cancer (Ringelhan et al. 2017). Cervical cancer risk is increased six times by HIV infection (Stelzle et al. 2021).

A recent WHO report from 2020 states that, with 2.26 million new cases, breast cancer is very common type. Lung cancer had 2.21 million cases, colon and rectal cancer showed 1.93 million cases, prostate cancer had 1.41 million cases, non-melanoma skin cancer had 1.20 million cases, and stomach cancer had 1.09 million cases. According to the same report, the leading cause of death in 2018 was in the sequences of lung cancer> colon and rectal cancer> liver

cancer or HCC> stomach cancer> breast cancer (685 000 deaths), (with their values, 1.80 million>916 000>830 000>769 000>685 000 deaths).

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Normal cells and neoplastic cells

Fundamental building blocks of a living organism is the normal cells.

When the death signal is sent, these cells divide or multiply to create new daughter cells, and then they grow, differentiate between acquiring appropriate phenotypic and functional traits, aging, and dying – a process called programmed cell death.

When this coordination is lost, the healthy cells develop into malignant ones.

The cells' DNA is the first source of the signal.

Cells proliferate uncontrollably and avoid the normal cell death phase when a gene mutation encodes cell cycle regulating proteins.

Cell mass or tumor formation results from unchecked cell division.

Unlike normal cells, cancerous cells initially spread to adjacent tissue areas and eventually, invade other locations, and can develop into a secondary tumor. This is linked to the new blood vessels formation in new tumor area (angiogenesis), which provide the mass of cells with nutrients and oxygen. The loss of a cancer cell's ability to differentiate is another characteristic. Less differentiated cancer cells that differ from normal cells in both phenotype and function are indicative of more aggressive malignancies. The descriptions of the phases of cancer development are described underneath.

Uncontrolled proliferation of neoplastic cells

One of the main characteristics of the onset and spread of cancer is persistent cell proliferation. The unchecked and ongoing random multiplication of cancer cells is the primary stage of cancer formation. By correctly reacting to the proliferation signals and preserving their typical phenotypic and function, normal cells regulate their rate of proliferation. Cancer cells, on the other hand, expand and divide uncontrollably because they genetically lose the ability to control their growth and begin to behave differently from their nearby non-cancerous counterparts. Cancer cells have a special trait that enables them to proliferate uncontrollably and survive longer than they would normally (Feitelson et al. 2015). The development and advancement of cancer, also known as carcinogenesis or oncogenesis, is a multi-step process.

Single-cell clonal growth and clonal selection are hallmarks of the evolution of cancer. The cell selection with progressively greater ability for cell division, and viability occurs, after cells begin to accumulate genetic alterations of the genes related to growth regulation and proliferation (Cooper 2000). The following describes the many stages of aberrant cell proliferation in cancer.

Genetic mutation and cancer genesis

The main antecedent to the development of cancer is a somatic mutation in the DNA (Brücher and Jamall 2016). Recent advances in whole genome sequencing analysis have made it possible to read the genomes of several cancer forms. The convenient core dogma's involvement is supported by all available evidence. Based on their effects and roles in the development of cancer, mutations in the cancer genome can be divided into a number of categories. Driver mutations are a group of mutations in the genes that directly impact oncogenesis and regulate the cellular proliferation, differentiation, development, and survival of cancer cells (Brown et al. 2019; Pon and Marra 2015; Bozic et al. 2010). These mutations favor clonal selection and expansion in the tumor microenvironment (Greaves and Maley 2012). Following the acquisition of driver mutations by cancer cells, a variety of random mutations known as passenger mutations accumulate within the genome of the cancer cell without contributing structurally or functionally to the cancer development and pregression (Stratton et al. 2009; Pon and Marra 2015). Cell death is encouraged and cell survival is hampered by another type of mutation. Negative selection prevents these mutations from becoming part of the cancer genome (Stratton et al. 2009).

Neoplastic cells deviate from the regulated cell cycle

The body's somatic cells transfer genetic material to their daughter cells through a carefully controlled cell cycle. Nonetheless, there are other checkpoints that coincide with the two primary cell cycle phases of interphase and mitosis (Barnum and O'Connell 2014). To put it briefly, following a full cell division, the newly formed two cells move into the G1 phase, which is a resting phase during which they acquire all the mediators required for DNA replication and move on to the subsequent S phase. In order to create sister chromatids, the DNA is copied during the S phase. To further prepare for cell division and double-check the

duplicate chromosomes for mistakes, the cell then enters the second resting state, known as G2. Additionally, mistakes are fixed prior to the next mitotic phase (M). Without receiving a suitable signal for the next division, the cells may occasionally remain in a quiescent phase and enter the G0 phase, which is frequently referred to as an extended G1 phase. The cell cycle is regulated by a number of crucial regulators, including cyclins and cyclin-dependent kinases (CDKs). These proteins' malfunction or hyperactivation causes these checkpoints to become dysregulated during the development of cancer. The majority of malignancies have dysregulated G1 to S phase regulatory cascades, which act as mitogenic signals for cancer growth (Matthews et al. 2022).

Tumor suppressor genes

The set of proteins that limit cell division and encourage programmed cell death is encoded by tumor-suppressor genes, which reduces the likelihood of tumor growth (Lee and Muller 2010). Tumor suppressor genes undergo mutations, knockdowns, or deactivations during oncogenesis. The first tumor suppressor gene is the retinoblastoma (RB) gene, which codes for a nuclear protein that mediates the cell cycle regulatory checkpoint at the G1 phase. Breast or ovarian cancer develops as a result of this gene mutation. Growth arrest, DNA repair, and apoptosis are all greatly aided by the transcription factor p53, one of the mostly known primary tumor suppressor genes (Mercadante and Kasi 2022). The *p53* gene has been implicated in 50% to 70% of all cancer cases. There is evidence of a mutation in the *p53* gene (Rivlin et al. 2011; Ozaki and Nakagawara 2011). The DNA repair protein that is encoded by BRCA2, another significant tumor suppressor gene, regulates cell division, cell death, and double-stranded DNA break repair. The pathophysiology of breast cancer is substantially linked to mutations in this gene (Saleem et al. 2020; Mersch et al. 2015).

Cancer cells metastasis: invasion, and angiogenesis

One of the main characteristics of malignancy is metastasis, which is derived from the Greek word methistanai, which means "a shift to a new place." It is the ability of cancer cells to spread throughout the body and the cells differentiate them from healthy somatic cells (Zeeshan and Mutahir 2017; Robert 2013; Dudjak 1992). Beyond their originating site, cancer cells can enter blood arteries and lymphatic nodes, move through them, and infiltrate nearby healthy tissues

to form another tumor mass. A highly coordinated series of minor events, commonly referred to as the "metastatic cascade," govern the entire process (Eslami et al. 2020). The metastatic cascade can be categorized into three main stages based on the order of events: invasion, intravasation, and extravasation (Jiang et al. 2015).

Invasion. The distinctive ability of malignant cancer cells to separate from their source or the main tumor mass due to a loss of cell-cell adhesion function and a dysregulation of cell-matrix interaction is what defines invasion. It is followed by an invasion of the surrounding cells' stroma. The following things happen throughout this process:

Decline in Cell-to-Cell Adhesion

Cell-to-cell adhesion is primarily mediated by tight junctions, adherens junctions, gap junctions, desmosomes, and integrins, which span from the apical to the basal membranes (Martin and Jiang 2009). Disruption of the structural proteins in these junctions can lead to cell-cell dissociation, loss of contact inhibition, and breakdown of the basement membrane. Structural proteins such as occludin, ZO-1, ZO-2, and claudin-7 are directly associated with cancer progression (Martin et al. 2010). Hepatocyte growth factor/scatter factor (HGF/SF), a cytokine released from stromal cells, plays a key role in the metastatic process, particularly in breast cancer development (Utoguchi et al. 1996; Martin et al. 2004). E-cadherin, a critical adherens junction molecule, is often downregulated or mutated in epithelial cancers (Cavallaro and Christofori 2004; Hsu et al. 2000). Desmosomal proteins, including PKP3, desmoglein 2, and desmoglein 3, have been found to be dysregulated in cancers like skin, prostate, lung, and head and neck cancers (Furukawa et al. 2005; Breuninger et al. 2010; Brennan and Mahoney 2009; Hsu et al. 2000), while desmocollin 2 is associated with colorectal cancers (Kolegraff et al. 2011). Furthermore, gap junction proteins such as connexin 43, integrins, and selectins are implicated in various cancer types (Bendas and Borsig 2012; Bonacquisti and Nguyen 2019).

Upon detaching from their primary site, cancer cells infiltrate the extracellular matrix (ECM), penetrate blood vessels, and enter the bloodstream in a process termed intravasation (Martin et al. 2013). They can later exit the bloodstream and colonize other tissues, a process known as extravasation (Martin et al. 2013).

Angiogenesis

During oncogenesis, the growth of the tumor mass is driven by cell division and metastasis. To support the growing tumor, new blood vessels are formed through angiogenesis (Nishida et al.

2006; Viallard and Larrivée 2017). Similarly, lymphangiogenesis refers to the formation of new lymphatic vessels (Nishida et al. 2006). Angiogenesis is regulated by both activators and inhibitors, with their balance playing a crucial role in cancer progression.

The upregulation of angiogenic activators, such as VEGF family proteins (VEGF-A-E), promotes the growth of blood vessels (VEGF-A, VEGF-B) and lymphatic vessels (VEGF-C, VEGF-D) by activating their respective receptors (Neufeld et al. 1999; Rafii and Skobe 2003; Mandriota et al. 2001). Additionally, hypoxia-inducible factor-1α (HIF-1α), which is induced under low oxygen conditions, enhances VEGF production and its receptor expression (Bottaro and Liotta, 2003). Other growth factors, such as tumor necrosis factor (TNF)-α, transforming growth factors (TGF)-α and β, and basic fibroblast growth factor (bFGF), also promote angiogenesis and contribute to cancer progression (Nishida et al. 2006). Angiogenesis inhibitors, including endostatin, angiostatin, thrombospondin, interferon, and tissue inhibitors of metalloproteinases (TIMP-1, -2, and -3), are often downregulated in tumors and may serve as potential therapeutic agents (Nishida et al. 2006).

Cancer Cell Differentiation

Cell division and proliferation are essential for cellular function and homeostasis. Cancer disrupts this balance, leading to uncontrolled cell growth. In contrast, differentiation refers to the process by which a cell adopts specific functional and phenotypic traits. Cancer cells often exhibit a loss of differentiation, a phenomenon known as anaplasia. The degree of differentiation can be assessed histologically and is indicative of tumor grade. Poorly differentiated cancers tend to be high-grade, exhibiting rapid proliferation and more aggressive behavior, while well-differentiated cancers are low-grade and less aggressive (Masic et al. 2008). Many cancers are also characterized by impaired apoptosis, making treatment challenging. Drugs that promote differentiation are being explored as an alternative strategy to induce cancer cell maturation and suppress uncontrolled growth.

Tumor Microenvironment (TME)

The TME consists of not only malignant tumor cells but also various stromal cells, immune cells, and extracellular matrix components. These elements interact to influence tumor growth and metastasis. Targeted therapies, including tyrosine kinase inhibitors and angiogenesis inhibitors, aim to modify the TME to control cancer progression. The TME is also influenced by immune cell activity, which can either promote or inhibit tumor growth (Baghban et al. 2020).

Immune Cells in TME

Immune cells within the TME can have both pro- and anti-tumor effects. Tumor cells can alter the TME by inducing changes in immune cell function, thereby promoting metastasis and local invasion. Immune cells in the TME include natural killer (NK) cells, B cells, macrophages, neutrophils, and fibroblasts. B cells, for example, can either support tumor growth by producing cytokines and antibodies or help limit tumor progression (Yuen et al. 2016; Kumar et al. 2018). NK cells play a role in detecting and killing tumor cells, although their effectiveness in the TME is often reduced (Paul and Lal 2017). Neutrophils can contribute to both tumor growth and immune responses, depending on their activation state (Masucci et al. 2019). Macrophages, particularly the M2 phenotype, promote tumor growth and immune suppression, whereas M1 macrophages assist in tumor cell destruction (Arnold et al. 2015). Cancer-associated fibroblasts are integral to the TME, promoting tumor growth, immune suppression, and extracellular matrix remodeling (Mao et al. 2021).

Extracellular Matrix (ECM) in TME

The ECM, composed of proteins like collagen, elastin, laminins, and proteoglycans, provides structural support and regulates cellular behavior within tissues. In cancer, ECM remodeling plays a key role in promoting tumor growth and metastasis. Dysregulation of ECM components, such as increased collagen cross-linking and proteolysis of elastin, creates a more rigid and invasive environment that supports tumor progression (Ozbek et al. 2010; Frantz et al. 2010; Kaczorowska et al. 2020). Additionally, proteoglycans like perlecan and syndecans influence tumor cell behavior and angiogenesis, further contributing to cancer progression (Couchman et al. 2012).

Cancer Cell-Mediated ECM Degradation

The degradation of the ECM is crucial for tumor cell metastasis and invasion. A group of enzymes, matrix metalloproteinases (MMPs), cause ECM breakdown, releasing bioactive fragments that support tumor growth and invasion (Kessenbrock et al. 2010; Brassart-Pasco et al. 2020). These enzymes generate matrikines, such as arrestins and VG-6, which play important roles in angiogenesis and cancer progression (Cabral-Pacheco et al. 2020; Brassart-Pasco et al. 2020).

TME-Related Immune Suppression

Tumor cells within the TME exploit various mechanisms to evade immune surveillance. Factors like CSF-1, IL-10, and IDO contribute to immune suppression by inhibiting immune cell activities (T lymphocytes and natural killer cells) (Mocellin et al. 2005; Li et al. 2017).

These immune evasion strategies complicate the effectiveness of immunotherapies, which aim to restore immune system function to target cancer cells.

CSF-1 (Colony-Stimulating Factor)

CSF-1 is crucial for the survival and function of macrophages in the TME. Elevated CSF-1 levels are associated with poor survival outcomes in several cancers, including breast cancer (Achkova et al. 2016). CSF-1 activates the CSF1R receptor, promoting macrophage recruitment to tumors (Aharinejad et al. 2013).

IL-10 (Interleukin-10)

IL-10, an anti-inflammatory cytokine, generates in immune cells. It suppresses immune responses by inhibiting Th1 activity and T cell cytotoxicity, allowing cancer cells to evade immune detection (Mocellin et al. 2005). Its production within the TME contributes to immune dysfunction and tumor progression.

Indoleamine 2,3-Dioxygenase (IDO)

IDO is an enzyme that degrades tryptophan, a crucial amino acid for T cell function, into kynurenine, which inhibits T cell proliferation and promotes immune tolerance (Godin-Ethier et al. 2011). Overexpression of IDO in tumors is associated with poor prognosis, as it impairs immune responses and facilitates tumor immune evasion (Li et al. 2017).

Vascular Endothelial Growth Factor (VEGF)

VEGF and its family members, including VEGF-A-D, are key regulators of angiogenesis in the TME. VEGF-A, in particular, promotes blood vessel formation by binding to its receptor VEGFR-2, thereby stimulating endothelial cell proliferation and survival (Neufeld et al. 1999). VEGF-C and VEGF-D, on the other hand, promote lymphangiogenesis by binding to VEGFR-3, contributing to tumor metastasis and lymphatic invasion (Rafii and Skobe 2003).

A protein called galectin-9 (Gal-9) is present in a lot of malignancies and is crucial for tumor immunity (Rabinovich et al. 2007). Unexpectedly, T cell weariness is brought on by the suppression of antitumor responses through TIM-3. This condition was first observed in vitro in people infected with HIV-1 (Jones et al. 2008) and subsequently in cancer patients (Gao et al. 2012). According to reports, TIM-3 expression is elevated in RCC patients' tumors and myeloid cells (Komohara et al. 2015). Its overexpression on CD8+T cells causes immune evasion at an early stage (Cai et al. 2016).

Inhibitors of immune checkpoints

An essential component of our immune system, the immunological checkpoint is required to reduce autoimmune inflammation. Receptors known as immune checkpoints are expressed on the surface of immune cells like T cells. They identify and bind to other partner proteins found on other cells, such as tumor cells or tumor-associated antigen-presenting cells. Checkpoint proteins and companion proteins work together to prevent T cells from destroying tumor cells, which ultimately paralyzes adaptive immunity against cancer. Immuno-check point inhibitors are used for immunotherapy that prevents checkpoint proteins from joining forces with their companion proteins, enabling the immune system to eliminate tumor cells and preventing cancer.

CTLA4 protein inhibits the checkpoint blocker; PD-1 and its companion protein PD-L1, which are recognized immune checkpoint blockers. Certain cancers suppress the T-cell response when there is an abundance of PD-L1.

CTLA-4

Several stimulatory signals are necessary for T-cell activation. Selectivity in T-cell activation is provided by TCR binding to MHC; however, additional co-stimulatory signals are necessary. The coherence of molecules like B7-1 (CD80) or B7-2 (CD86) on the APC with CD28 molecules on the T cell triggers signaling within the T cell. Increased consumption of CD28:B7-1/2 binding enhances energy metabolism, upregulates cell survival genes, and stimulates T-cell proliferation, survival, and diversity through growth cytokines like interleukin-2 (IL-2) production. A homolog of CD28, CTLA-4 has a significantly greater affinity for B7 for cohering. The competitive coherence can prevent the co-stimulatory signal that is often produced by CD28-B7 binding, even if binding of CTLA 4 to B7 does not provide any stimulatory signals, in contrast to CD28 (Parry et al. 2005; Egen et al. 2002; Chambers et al. 2001). The activation or anergy of such a T cell depends on the proportionate amount of CD28. B7 binding against CTLA-4: B7 binding. In fact, some studies suggest that CTLA4-B7 binding may produce inhibitory signals that balance the stimulatory signals generated by TCR-MHC and CD28-B7 binding. (Masteller et al. 2000; Fallarino et al. 1998).

When naive T lymphocytes are at rest, CTLA-4 is mostly found in the intracellular compartment. Signals triggered by TCRs and CD28: B7 cohering results in the exocytosis of CTLA-4-embodying vesicles, which upregulates CTLA-4 on the cell surface. Stronger TCR signaling causes more CTLA-4 to translocate to the cell surface. In the case of a net negative signal via CTLA-4: B7 cohering, reduction of IL-2 production and cell cycle progression hinders full activation of T cells (Krummel et al. 2008).

PD-1 cascade

PD-1 belongs to B7/CD28 co-stimulatory receptor family. Through cohering to its ligands, namely programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2), it activates T cells (Keir et al. 2008). Similar to CTLA-4 signaling, PD-1 cohering decreases T-cell survival and inhibits the generation of IL-2, interferon-γ (IFN-γ), tumor necrosis factor-α, and T-cell proliferation (Keir et al. 2008). While PD-1 is mostly expressed on activated T cells, myeloid cells, B cells, and CTLA-4 is solely found on T cells, and both cohering have similar detrimental effects on T-cell activity (Keir et al. 2008). Leukocytes, non-hematopoietic cells, and non-lymphoid tissues are the primary sites of PD-L1 expression. In rare cases, inflammatory cytokines or tumorigenic signaling pathways can also activate PD-L1 on parenchymal cells (Chen 2004). PD-L1 expression is seen in many different types of tumors and is linked to a higher number of tumor-infiltrating lymphocytes (TILs) and a worse prognosis (Taube et al. 2014; Hino et al. 2010; Zou et al. 2008). Although it depends on the local microenvironment, PD-L2 is mostly expressed on dendritic cells and monocytes. (Rozali et al. 2012). T-cells while engaging in an effector T-cell response, PD-1 and its ligands cooperate to decrease the magnitude of the immune response. As with CTLA-4 inhibition, this results in a more limited spectrum of T-cell activation (Ott et al. 2013).

Metabolism interaction and TME

Individual cell survival, proliferation, and death interact intricately to generate the tumor microenvironment (TME), a collection of altered cells and healthy tissue components that either support or oppose tumor sustenance and advancement. Given the enormous number of rapidly proliferating and dividing cells found in tumors, the amount of metabolic resources allocated to each cell is significantly reduced (DeBerardinis and Chandel, 2016). Furthermore,

it is very difficult for nutrients and oxygen to freely percolate through the tumor mass and disperse uniformly to each cell due to the dense packing of cells within the small volume of the tumor (Bull et al. 2020). Therefore, in order for the cells to live on such a limited amount of food, the tumor must undergo a comprehensive metabolic reprogramming. The auxiliary cells in the TME, in addition to the tumor cells, also experience significant metabolic adaptation to deal with the hypoxic, nutrient-depleted tumor milieu. Furthermore, during the actively growing and metastatic phases, constitutive metabolic interaction between the tumor cells and the auxiliary cells supports the tumor progression (Wei et al. 2021).

Metabolic reprogramming of neoplastic cells

Central carbon metabolism is one of the main routes impacted when a cell undergoes neoplastic transformation. In order to reduce coenzymes like nicotinamide adenine dinucleotide (NAD+), flavin adenine dinucleotide (FAD), etc., pyruvate, the by-products of glycolytic oxidation of glucose enters the mitochondria and produces electrons. These electrons are then directed through the electron transport system to encourage oxidative phosphorylation (OXPHOS). Because the mitochondrial portion of this route depends on molecular oxygen, this entire chain is crucial (Porporato et al. 2018). Oxygen frequently becomes a limiting factor for cells in the highly competitive tumor microenvironment, particularly in the tumor's core (Petrova et al. 2018).

Interestingly, lactate has been associated with the inhibition of intracellular innate immune checkpoints in mimetic circumstances and oncoviral infections (Zhou et al. 2021; Zhang et al. 2019). Tumor cells maintain a high rate of the pentose phosphate pathway (PPP), which produces massive amounts of ribo- and deoxyribonucleotide precursors necessary for their rapid DNA replication, in addition to an increased rate of glycolysis (Patra and Hay, 2014). To promote glucose diffusion into the cell, tumor cells express more glucose transporters since a significant amount of glucose is required to initiate both glycolysis and PPP (Macheda et al. 2005).

Another essential characteristic of cancer is the dysregulation of lipid metabolism. Surface transporters for fatty acids (FA) are frequently expressed in excessively high levels in tumor cells (Kim et al. 2019). According to several findings, colorectal cancer cells prefer to

absorb polyunsaturated fatty acids (Mika et al. 2020). Increased β-oxidation of FA occurs in the peroxisome rather than the mitochondria as a result of enhanced FA absorption (Zha et al. 2005). In addition to its metabolic functions, the resulting acetyl CoA is essential for controlling gene expression by histone modification and specific transcription factor recruitment (Martinez-Reyes and Chandel, 2018). Additionally, tumor cells have remodeled machinery that increases fatty acid production (Fhu and Ali 2020). However, in the neoplastic situation, the normal distribution of the lipids in various subcellular compartments is significantly altered. For instance, a number of malignancies are linked to inadequate amounts of cardiolipin, which causes the integrity and permeability of the mitochondrial membrane to deform (Ahmadpour et al. 2020).

In addition to nucleic acid precursors, which are necessary for cancer cells to proliferate rapidly, non-essential amino acids (NEAA) are one of the main sources of cellular nitrogen sinks in tumor cells. Most tumor cells upregulate the absorption and production of NEAAs because of the local depletion of these molecules in primary and metastasizing tumors (Lieu et al. 2020). The preservation of stemness and the differentiation of cancer cells depend on glutamine, one of the essential amino acids required for cellular maintenance (Jariyal et al. 2021). Curiously, despite its incapacity to function as a substrate for glutamine production, asparagine steps in as an important amino acid at times of glutamine scarcity, demonstrating indirect metabolic complementarity between related amino acids (Pavlova et al. 2018). There are significant changes in metal ion homeostasis in tumor cells when compared to normal cells. Key glycolytic enzyme location and catalytic performance are disrupted by markedly altered calcium dynamics along the extracellular space-cytosol-ER axis, which impacts glycolysis in cancer cells (Dejos et al. 2020).

Cancer cells take in, digest, and export iron—another cation of biological significance—at unusually high rates, as shown by the expression levels of the appropriate genes. By combining the leakage of electrons from the former mitochondrial membrane with such high loads of free iron within the cell, a Fenton chemistry-based mechanism causes an excess of reactive oxygen species (ROS) (Bystrom et al. 2014). The cellular structure is severely damaged by these reactive radicals, which exacerbate carcinogenesis by causing lipid peroxidation and irreversible DNA damage (Juan et al. 2021). Additionally, ROS promotes anaerobic glycolysis, which leads to the "Warburg effect"—a sudden spike in lactate production (Liberti and

Locasale 2016). Remarkably, ROS's biological actions typically exhibit a hormetic tendency, which helps explain its phase-dependent activities throughout cancer (Maryanovich and Gross 2013).

Immune cells that infiltrate tumors and undergo metabolic changes

During their pre-tumoral and intratumoral phases, T cells—one of the most common immune cells in the TME—go through a severe metabolic rewiring process. T cell receptor (TCR) signaling-mediated antigenic priming increases the rate of aerobic glycolysis, whereas naive T cells maintain a basal level of glycolysis (Chapman et al. 2020). However, a preference for increased mitochondrial metabolism, specifically OXPHOS and FA catabolism, is induced upon differentiation into the regulatory T cell phenotype (Michalek et al. 2011). The role of this master metabolic regulator in mediating the metabolic changes during memory T cell growth has been established by studies focusing on the biochemical activities of mTOR (mechanistic target of Rapamycin) in the context of T cell differentiation (Araki et al. 2009). On the other hand, prolonged T cell activation in the hypoxic TME causes oxidative burst and mitochondrial malfunction, which ultimately results in T cell fatigue and apoptosis (Scharping et al. 2021). Interestingly, comparable metabolic shift patterns are seen in macrophages throughout their differential polarization (Puthenveetil and Dubey 2020) and NK cells during their activation and differentiation (Terrén et al. 2019). A notable modification in the TME myeloid compartment pertains to the metabolic pathways of amino acids. Naive macrophages choose M1/M2 polarization due to competition for arginine between two enzymes, arginase and inducible nitric oxide synthase (iNOS) (Rath et al. 2014). Its degradative effects frequently compound with those of the reactive nitrogen species (RNS), resulting in even more harmful free radicals like peroxynitrite (Espey et al. 2000). Deaminated and transaminated glutamate products drive the TCA cycle by anaplerotic complementation (Zhao et al. 2019), although tryptophan catabolites like kynurenine trigger their producer cell activation dynamics (Platten et al. 2012). MDSCs actively import sulfur-containing amino acids, like cysteine and cysteine, and reduce the expression levels of their exporters because the TME levels of these amino acids are far below a tolerable amount (Srivastava et al. 2010).

Metabolic interplay in the TME

The normoxic tumor cells and a number of normal cells, including fibroblasts, macrophages, etc., absorb and use lactate into their extracellular milieu as a substrate for sustenance (Lacruz-López et al. 2019). Tumor cells actively release tryptophan-derived Kynurenine to suppress the effector response and cause intratumoral T lymphocytes to become anergic (Rad Pour et al. 2019). The ectonucleotidases CD39 and CD73, which are expressed on tumor cells, hydrolyze ATP to produce adenosine, a strong inhibitor of T- and NK-cell activation (Häusler et al. 2011). However, depending on the local redox state, nitric oxide produced into the tumor milieu by classically activated macrophages, dendritic cells, etc., either stimulates or inhibits tumor growth (Hickok and Thomas 2010). By creating a gradient of minerals inside the tumor borders, competition for minerals like iron (Pfeifhofer-Obermair et al. 2018) and copper (Lelièvre et al. 2020) systemizes the TME. The metabolic propensity of the involved cells also affects a number of juxtacrine and paracrine connections. The rate of glycolysis directly affects the expression of the co-inhibitory protein PD-L1 on tumor cells (Jiang et al. 2019). By preventing p65 from being recruited on the IL-9 promoter, cholesterol inhibits the production of IL-9 in CD8+ T cells (Ma et al. 2018). Another important factor in the metabolic changes inside the TME is the directed transfer of metabolites, metabolic enzymes, and solute transporters from one cell to another via released exosomes (Yang et al. 2020). More than two actors are frequently involved in the intra-tumoral metabolic interaction. One of the best instances of this phenomenon is the inhibition of T cell proliferation by tumor mesenchymal stem cells, which prevent cysteine export from dendritic cells (Ghosh et al. 2016). Each and every cell in the tumor has a different mix of oxygen and nutrients, which is primarily represented in its metabolic landscape (Xiao et al. 2019). Furthermore, distinct expression patterns of the essential metabolic regulatory modules are present in various cancer grades and stages (Mukherjee et al. 2021).

Metastasis and TME

The growth and survival of cancerous cells depend on the tumor microenvironment (TME). Metastasis is one of the main causes of cancer-related deaths, even with new developments in cancer management and treatment techniques. When cancer cells spread, they break off from the original tumor location, enter the bloodstream, and settle in other organs to create secondary foci. The extracellular matrix (ECM), which is made up of structural elements including

collagen, laminin, and hyaluronan, as well as various immune cells, endothelial cells, and cancer-associated fibroblasts, make up the TME. The cellular and structural elements of TME interact intra-tumorally to cause immune cell evasion, aggressiveness, invasion, and cancer cell circulation.

The modulatory function of immune cells in the spread of cancer

By entering the tumor or affecting the systemic microenvironment, immune cells, which are widely recognized as the fundamental biological component of TM, have a significant impact on cancer cells at different stages of invasion-metastasis (Kitamura et al. 2015). Tumor-associated macrophages (TAMs) are created when macrophages are drawn to the tumor site and into the TME by chemokines released by stromal and cancer cells. The two subtypes of TAMs, M1 and M2, have opposing roles based on their polarization. Classically activated TAMs of the M1 type stimulate the immune system by generating interferon (IFN)-γ and have anti-tumoral and pro-inflammatory properties. However, by producing IL-1β, inducing angiogenesis, and encouraging tumor cells to release MMPs, which cause cancer to progress by rupturing the extracellular matrix and basement membrane, M2-type (alternatively activated) TAMs exhibit pro-tumorigenic activity and perform an immunosuppressive role (Whipple and Chery 2015). At intravasation sites, TMEM is the term used to describe the close proximity of endothelial cells, perivascular TAMs, and cancer cells (Robinson et al. 2009). These tumor cells consequently acquire mesenchymal potentials and lose epithelial traits, increasing their ability to invade and spread to other cancer cells (Thiery et al. 2009). Glypican-expressing cancer cells in hepatocellular carcinoma (HCC) interact with TAMs in the tumor microenvironment by secreting TGF-β, PDGF, VEGF, chemokine, and M-CSF (Macrophage colony-stimulating factor) (Gupta et al. 2014). TAMs cause pancreatic cancer cells to have increased fibroblastic shape, proliferation, and motility by upregulating various mesenchymal markers like vimentin and snail, activating proteolytically active MMPs like MMP-2 and MMP-9, and lowering E-cadherin levels (Liu et al. 2013).

The function of mesenchymal stem cells (MSCs) in controlling metastasis

MSCs participate in different phases of tumor growth, causing the initial tumor site to develop stem-like characteristics, invasion, and the ability to spread (Sarvaiya et al. 2013). C-X-C and

C-C chemokines, among others, are secreted by MSCs and encourage tumor cell migration to the secondary location. MSCs increase the expression of CXCR4, and overexpressed CXCR4 contributes to the migration of leukemic cells to bone marrow, which leads to chemotherapeutic resistance mediated by the stroma (Jin et al. 2008).

Exosomes enhance breast cancer cellular ability to migrate by upregulating the TGF-β and WNT pathways, which target genes such as *Axin2 Dkk-1* (Lin et al. 2013). Bone marrow-derived MSCs can facilitate migration by generating exosomes that contain various chemotactic proteins, including MCP-1 and MCP-2 (Vanderkerken et al. 2002).

How the cancer-associated fibroblast (CAF) controls metastasis.

CAFs are distinct from other sub-cell types due to the classic CAF biomarkers, which include integrin β1 (CD29), α-smooth muscle actin, and fibroblast activation protein (FAP) (α-SMA) (Puré et al. 2018; Costa et al. 2018). The fact that FAP, CD29, and α-SMA are not exclusive to CAFs is notable. Through the production of collagen and fibronectin, the activation of MMPs, and the elevation of VEGF levels, CAFs facilitate ECM remodeling. This results in a reorganization of the matrix that controls the directional migration of cancerous cells with CAFs (Gonzalez-Avila et al. 2019; Kati and Vaheri, 2010; Eck et al. 2009; Murphy et al. 2008; Gaggioli et al. 2007). CAF-derived laminin interacts with integrin α6β4 to stimulate cervical cancer cell motility. CAF is a key player in the stroma that modifies the ECM shape and content (Fullár et al. 2015; Faouzi et al. 1999).

Endothelial cell (EC) function in metastasis

ECs significantly affect the biochemical gradients in the TME and the supply of inflammatory cells via changing blood flow. Loosely connected lymphatic endothelial cells (LECs) may be responsible for the easy access of the lymphatic channels that support tumor development at the secondary location, perhaps promoting metastasis (Smolarczyk et al. 2021; Rankin et al. 2016). According to Albrecht et al. 2011, LECs produce a variety of chemoattractants, namely CCL21 and SDF-1, that attach to the CCR7 and CXCR4 receptors, respectively, present in cancer cells. When the PI3K/AKT pathway is activated in tumor cells, VEGF release rises and cell survival is controlled. There are numerous cellular functions that include the Ras family

(H-Ras, N-Ras, and K-Ras). Increased expression of VEGF, which controls cell proliferation, is associated with mutations in K-Ras and H-Ras.

Clinical challenges of cancer stem cells

Among the most challenging candidates for therapeutic targeting are cancer stem cells (CSCs). Because of their incredibly low rate of proliferation, CSCs are not completely cleared by the majority of chemotherapy and radiation treatments, which are designed to eradicate quickly dividing cancer cells (Zhao et al. 2016). Furthermore, despite the massive mutation load, CSCs are certain to survive thanks to their incredibly effective arsenal of DNA repair enzymes (Valencia-González et al. 2009). Additionally, the cells boost the expression levels of drug efflux pumps (Moitra et al. 2011), anti-apoptotic proteins (Lauria et al. 1997; Static et al. 2008), and drug-rich setting in cancer cells while chemotherapy is in progress. Due to the aforementioned causes, CSCs are a major contributor to the partial remission and recurrence of numerous cancer types (Yu et al. 2012). Because the surface proteome of CSCs overlaps with that of healthy, non-cancerous stem cells, biomarker-targeted therapies targeting CSCs are also not always effective. An autophagy inhibitor called salinomycin, for instance, shown notable therapeutic activity against breast cancer CSCs (Gupta et al. 2009), but not in CSCs that depend on acidic lysosomes for cellular recycling instead of autophagosomes (Jangamreddy et al. 2013). For instance, occurrences of leukemic cells from bone marrow homing and post-therapy relapse were significantly reduced by monoclonal antibodies against IL3Rα, a marker that is exclusively expressed by AML (acute myeloid leukaemia) stem cells and not by healthy stem cells (Jin et al. 2009; Jordan et al. 2000). Apart from that, precision therapy in both cancer type-specific and pre-cancer modes would be made possible by identifying transcriptomic and proteomic characteristics unique to a single CSC and establishing links with their biological condition.

Extracellular TME and cancer spread

Extracellular pH

The high demand for the bio-synthetic product causes tumor cells to switch to anaerobic glycolysis, which turns glucose into lactate. Excess lactate is subsequently released in the tumor

microenvironment (TME), resulting in an acidic environment (Stubbs et al. 2000). By protonating many histidine residues, acidic extracellular pH activates GPR4, TDAG8, and OGR1 receptors, which may be involved in tumor formation, inflammation, metastasis, and angiogenesis (Ludwig et al. 2003). Additionally, acidic pH increases the activation of some lysosomal enzymes and certain genes of pro-metastatic factors via an intracellular signaling system (Rofstad et al. 2006). Blood vessel growth is aided by VEGF receptor expression, which is regulated by GPR4. Additionally, GPR4 impacts blood vessel stability and integrity (Wyder et al. 2011). TDAG8 promotes cell apoptosis evasion when acidosis is present, and overexpression of this gene has been shown to transform immortalized mammary epithelial cells (Sin et al. 2004).

Hypoxic microclimate

In order for cancer cells to survive and proliferate, hypoxia damages the TME and applies selection pressure that requires them to make adaptive genetic or epigenetic changes (Dhani et al. 2015; Richard et al. 2007). Levels of hypoxia-inducible factors (HIFs) rise during hypoxia in TME and are crucial for cancer cellular ability to survive in hypoxic environments (Lu et al. 2010). HIF works with its coactivator CBP/p300 to activate target gene promoters by attaching to hypoxia response elements (Wu et al. 2013).

The Complex Relationship Between Cancer and the Microbiota

Microbial communities have been shown to have immune-stimulatory effects on tumor tissues (Bessell et al. 2020; Kim et al. 2017), while there are also reports suggesting microbial immunosuppressive roles (Pushalkar et al. 2018; Kostic et al. 2013). According to Jain et al. (2021), many of these effects may result from alterations in systemic hematopoiesis rather than being directly linked to immune cell dynamics within the tumor itself. Additionally, recent studies have found that the microbiota within the tumor microenvironment (TME) may influence tumor progression by interacting with crucial physiological pathways, such as p53 signaling (Dong et al. 2022) or cytoskeletal rearrangement (Fu et al. 2022). Moreover, microbial metabolites have been shown to have variable impacts on the malignant traits of tumors (Rossi et al. 2020). Antibiotic treatments, which disrupt the native microbiota, have led to inconsistent outcomes in cancer therapies (Gao et al. 2020).

The Cancer Microbiome

Solid tumors, composed of various cellular and subcellular components, also harbor distinct microbial communities specific to the tumor type (Zhao and Hu, 2020). These microorganisms are typically found within the intratumoral spaces, including those inhabited by immune cells and cancer cells (Nejman et al. 2020). Beyond the well-known oncoviruses that promote host cell transformation (Cao and Li, 2018), various bacterial species (Cummins and Tangney 2013) and other microorganisms (Vennervald and Polman 2009) have been found in cancerous tissues. The microbial load within tumors varies significantly, ranging from 14% in melanoma to over 60% in breast and pancreatic cancers (Nejman et al. 2020), raising concerns about the active roles these microbes might play in tumor growth and metastasis.

Potential Theranostic Applications of the Microbiome in Cancer

Disruptions to the microbiome may offer valuable insights into the mechanisms and potential adverse effects of cancer treatments. The accumulation of such data could pave the way for personalized, targeted probiotic therapies against cancer (Śliżewska et al. 2020).

Targeting the Tumor Microenvironment (TME)

The TME comprises both soluble elements such as interstitial fluids, cytokines, and metabolites, as well as non-soluble components like the extracellular matrix (ECM) (Benesch et al. 2017; Wang et al. 2017; Quail and Joyce 2013; Fan et al. 2012). The TME plays a pivotal role in tumor initiation, metastasis, and resistance to treatment (Wang et al. 2017; Pickup et al. 2014; Gilkes et al. 2014). Targeting the TME, particularly through small molecule inhibitors, presents a promising therapeutic strategy, as these compounds can rapidly penetrate the TME (Zhong et al. 2020).

Targeting the Extracellular Matrix (ECM)

The ECM, a three-dimensional scaffold composed of collagen, elastin, fibronectin, and other molecules, helps maintain tissue structure and function (Willumsen et al. 2018; Pickup et al. 2014; Otranto et al. 2012). The composition of the ECM can significantly impact tumor prognosis (Hui and Chen 2015). Elevated ECM density and stiffness, particularly due to increased collagen production, result from factors such as tumor cell heterogeneity, poor tissue oxygenation, or heightened inflammation (Willumsen et al. 2018; Hui and Chen 2015). The ECM facilitates various cancer-promoting processes, including cell proliferation, angiogenesis, invasion, and metastasis (Pickup et al. 2014). FDA-approved angiotensin II receptor blockers, such as Losartan and Valsartan, have been shown to enhance chemotherapy delivery and reduce collagen production, leading to decreased tumor desmoplasia (Coulson et al. 2017; Diop-Frimpong et al. 2011). Additionally, ronespartat (SST0001), a heparanase inhibitor, has

shown promise in clinical trials for treating multiple myeloma (Cassinelli et al. 2013; Ritchie et al. 2011).

Focusing on the Acidic TME and Hypoxia

The TME's classification depends on whether it experiences acute or chronic hypoxia (Vaupel and Mayer 2014). Acute hypoxia occurs due to transient perfusion issues, whereas chronic hypoxia arises from factors like tissue oxygen diffusion limitations (Vaupel and Mayer 2014). The hypoxia-inducible factor 1 (HIF-1) pathway plays a central role under hypoxic conditions, driving transcriptional changes that facilitate tumor growth and survival (Ziello et al. 2007; Duffy et al. 2010; Masoud and Li 2015). The acidic environment of the TME, typically lower than the normal pH of 7.4, helps protect tumor cells by impairing immune cell function and chemotherapy efficacy (Webb et al. 2011; Netea-Maier et al. 2018). Strategies targeting TME acidification, such as using carbonic anhydrase inhibitors combined with chemotherapy, are being explored in clinical trials for cancers like small-cell lung cancer and pancreatic cancer (Roma-Rodrigues et al. 2019).

Targeting Pericytes and Endothelial Cells

Vascular endothelial growth factor A (VEGFA) induces angiogenesis upon binding to VEGF receptor 2 (VEGFR-2) on endothelial cells, promoting tumor vascularization (Klein 2018; Harrell et al. 2018; De Palma et al. 2017). Pericytes, which support endothelial cells, release growth factors that regulate blood vessel stability and endothelial cell survival (De Palma et al. 2017; Viallard and Larrivée 2017). Elevated VEGFA in the TME promotes neo-angiogenesis, resulting in leaky and poorly formed blood vessels (Klein, 2018; De Palma et al. 2017). Antiangiogenic therapies, such as the FDA-approved bevacizumab, target VEGF signaling to inhibit tumor vascularization (Fukumura and Jain 2007). Other multi-targeted tyrosine kinase inhibitors, like Pazopanib, are used to treat various cancers by disrupting angiogenesis (Noda et al. 2019; Nakano et al. 2019).

Chronic Inflammation in the TME

Tumor-associated macrophages (TAMs) contribute to chronic inflammation within the TME by releasing pro-inflammatory cytokines (Netea-Maier et al. 2018; Na et al. 2018). This persistent inflammation can drive carcinogenesis (Mantovani et al. 2018; Tashireva et al. 2017). Inflammatory modulators such as Anakinra, an FDA-approved IL-1 receptor antagonist, have shown promise in reducing bone metastases in breast cancer (Tulotta and Ottewell 2018). The anti-inflammatory drug canakinumab reduced inflammation and improved cancer progress in patients (Ridker et al. 2017).

Targeting Cancer-Associated Fibroblasts (CAFs)

Fibroblasts within the TME contribute to tumor progression by secreting growth factors that enhance cancer cell survival and invasion (Ray and Cleary, 2017). Cancer-associated fibroblasts (CAFs), which arise from mesenchymal stromal cells, play a central role in this process (Lamprecht et al. 2018). Targeting CAFs through novel drugs like conophylline, which has shown effectiveness in resistant pancreatic cancers, is an active area of research (Ishii et al. 2019). Developing small-molecule inhibitors and utilizing gene manipulation techniques, such as antisense oligomers, holds promise for disrupting CAF activity and improving cancer treatment (Mukherjee et al. 2005; Sharma and Allison 2015).

According to the results of the next-generation sequencing (NGS) investigation, 80% of HBV-positive HCC cases had the incorporation of the HBV genome. Additionally, compared to non-tumor tissues, there was a greater correlation with tumor tissues (Nault et al. 2013). In HBV-positive tumors, three cancer-associated genes—cyclin E1 (*CCNE1*), mixed-lineage leukemia 4 (*MLL4*), and telomerase reverse transcriptase (TERT)—were commonly found at the integration sites, indicating a very strong correlation between integration sites and hepatic neoplasia. According to published research, over 50% of HCC tissues contained a TERT promoter mutation (Nault et al. 2013; Sung et al. 2012). According to a large body of data, HBx plays important roles in the development of HCC through a variety of cellular and molecular processes (Bell et al. 2015; Nault et al. 2013; Sung et al. 2012; Zhang et al. 2012).

Patients over 50 with diabetes or obesity have been found to have non-alcoholic steatohepatitis (NASH), a form of NAFLD characterized by progressive fibrosis. The annual progression rate of NASH to HCC is 0.5% (Younossi et al. 2016a). Obesity and diabetes are two chronic metabolic diseases that dramatically raise the risk of HCC. Because the liver is essential for the metabolism of glucose, diabetes mellitus directly affects the liver, leading to cirrhosis, fatty liver, chronic hepatitis, and liver failure (Yang et al. 2017a; Younossi 2016b; White et al. 2012). According to the research, diabetes increases the incidence of HCC by two to three times and is recognized as an independent risk factor for the disease (El-Serag et al. 2006; Welzel et al. 2013). Numerous studies have indicated in the literature (Mittal et al. 2016; Huang et al. 2018; West et al. 2017) that if the anti-inflammatory pathway cascade is not controlled, the pleiotropic impact of insulin loses control over cellular proliferation, which leads to the induction of carcinogenesis. Additionally, cellular proliferation can be increased by insulin-like growth factor, whereas apoptosis can be downregulated by insulin receptor substrate I

(Yoshimoto et al. 2013; Park et al. 2010; Balkau et al. 2001). Hepatocytes may undergo malignant transformation and induce HCC as a result of obesity and a number of hepatobiliary disorders, including NAFLD, steatosis, and cryptogenic cirrhosis (Reddy and Rao 2006; Callee et al. 2003). By alone, obesity increases HCC risk by 1.5-4 times. According to Larson and Wolk (2007), the relative risks of HCC in obese patients and overweight subjects were 189% and 117%, respectively. The majority of cases of HCC-NAFLD have been reported in males. Male-developed HCC has far less cirrhosis and fibrosis, and half of the patients with HCC-NAFLD had no cirrhosis (Monsour et al. 2013; Turati et al. 2014). Compared to HCV-related HCC, tumors had a considerably lower AFP level and an enhanced des-γ-carboxy prothrombin level (Wakai et al. 2011; Tokushige et al. 2010).

Disease risk factors are quantitatively assessed based on the attributable population fraction (PAF). The PAF was conducted after a population-based study with 6,991 HCC patients older than 68 years was evaluated (Welzel et al. 2013). The study's conclusions showed that a 40% decrease in HCC occurrences occurred when diabetes and obesity were eradicated. After excluding HCV and other variables, the drop percentage was significantly higher. Few high-quality population-based studies have been conducted to examine the relationship between NAFLD and HCC. However, the results of the initial studies indicate that the focus for successful care and prevention of HCC would be on accurately assessing distinctive metabolic syndromes (Marrero et al. 2018).

Alcohol abuse is a major worldwide concern since it causes cirrhosis, fatty liver, alcoholic steatohepatitis (ASH), and ultimately, HCC (Schwartz and Reinus 2012). According to Neuman et al. (2015), long-term alcohol use activates cytochrome P450 2E1 (CYP2E1), a part of the cytochrome P450 mixed-function oxidase system, which affects a variety of biological processes, including increased alcohol metabolism, oxidative stress, hepatotoxicity, and collaboration between various medications, xenobiotics, and carcinogens. Alcohol metabolism produces aldehyde, which is a key factor in oxidative stress and the ensuing liver damage (Yoon 2018). Each year, 20–25% of instances of HCC are caused by alcoholic liver disease (ALD), and 1.3–3% are caused by alcoholic cirrhosis. Race and sex are important risk factors for HCC, with the PAF of ALD as a risk factor ranging from 13-23% (Massarweh and El-Serag 2017; Welzel et al. 2013). The presence of viral hepatitis specifically intensifies alcohol as a risk factor (independent type) for HCC (Marrero et al. 2018).

According to Yang et al. (2019) and Gouas et al. (2009), aflatoxin is a powerful mycotoxin that can contaminate a wide range of staple cereals and oilseeds and has high hepatocarcinogenic effects. Higher occurrences of HCC are found in areas where aflatoxins are widely present (Gouas et al. 2009).

Aristolicia and Asarum are two plants that grow all over the world and are the source of aristolochic acid (AA), a highly carcinogenic chemical (Arlt et al. 2002). The next-generation sequencing (NGS) study's findings demonstrated that a sizeable portion of HCC patients with Asian ancestry, particularly those from China, Taiwan, Vietnam, and South East Asia, had significant mutation rates with comparable patterns and traits to those that happened after being exposed to AA. 78%, 47%, 29%, 13%, 2.7%, 4.8%, and 1.7% of HCC patients from Taiwan, China, Southeast Asia, Korea, Japan, North America, and Europe, respectively, exhibited the signature mutational characters of AA, according to the results of a large study that included 1400 patients (Chen et al. 2018; Ng et al. 2017; Rosenquist and Grollman 2016; Hsieh et al. 2008).

According to a population-based cohort study with patients with hereditary hemochromatosis and 5973 members of their first-degree relatives, 62 patients developed HCC with a standardized incidence ratio of 21 (95% CI), 16-22. Thalassemia, an iron overload state, has no direct correlation with HCC, but it has been observed that Thalassemic individuals have a high prevalence of HCV, which may eventually increase their risk of HCC. Drinking beer in non-galvanized steel drums increases the risk of HCC compared to storing it in iron, which is at least ten times higher than that of regular iron stores. Men were more likely than women to acquire HCC, and there is no incidence risk of nonhepatic malignant transformation. One of the recognized risk factors for HCC is cirrhosis resulting from primary biliary cholangitis (PBC). According to a three-year research, the incidence of HCC was 5.9% in 273 patients with cirrhosis that developed from PBC. (Marrero et al. 2018).

According to Antoury et al. (2015), a prospective study with a median follow-up period of 5.2 years and cirrhosis patients resulting from alpha-1 antitrypsin deficiency found that the annual incidence of HCC was 0.9%.

HCC pathogenesis

With the use of established molecular and histological markers, characteristic, well-defined, and verified aspects of the initiation and developing phase of HCC have not yet been identified (Sia et al. 2017; Aravalli et al. 2013). Numerous pieces of evidence in the literature indicate that hepatic neoplastic transformation and, ultimately, the development of HCC are caused by the ongoing accumulation of mutations and genetic alterations in preneoplastic hepatocytes (Chakraborty et al. 2020; El-Serag and Rudolph 2007; Farazi and DePinho 2006). More often than not, the tumor is seen with several lesions rather than just one. While the cells of poorly differentiated tumors are large and difficult to differentiate from metastatic tumors of various origins, the neoplastic cells of well-differentiated tumors exhibit the properties of normal hepatocytes (Sia et al. 2017; Aravalli et al. 2013). Hepatocytes and cholangiocytes, the hepatic parenchymal cells, and Kupffer cells, stellate cells, endothelial cells, nonparenchymal cells, the fibroblasts are the fundamental components of the hepatic structure (Stranger 2015; Knouse et al. 2014). There are disagreements over the presence of stem cells in the adult liver. Liver-specific natural killer cells (NK cells) and intrahepatic lymphocytes are seen in the sinusoidal lumen and perisinusoidal area of Dizzee. The majority of the hepatic mass (60–80%) is made up of hepatocytes. The liver's architectural arrangement shows that the lobules are formed by the coordinated assembly of cells. Functional zones or areas are created by further differentiating lobules. Liver zones are crucial components, particularly for hepatocytes, as they significantly impact hepatocyte activity without impairing phenotypic. The majority of hepatocytes are polyploid (4N, 8N, etc.). According to Stranger (2015) and Knouse et al. (2014), 90% of the liver in mice and 50% of the liver in humans are made up of polyploid cells. Kupffer cell and hepatic stellate cells (HSCs) activate to response against toxic chemical mediated insult and the immunological response follows the causes inflammation in the liver and necrosis. During this phase, cirrhosis and liver fibrosis may develop (Severi et al. 2010). The primary stage of fibrosis, cirrhosis, is characterized by several hallmarks, including a change in blood flow, a significant risk of liver failure, and distortion of the liver parenchyma, septae, and nodule formation. Due to its significantly elevated mortality and morbidity, cirrhosis becomes the single most significant risk factor for the development of HCC (Roskams and Kojiro 2010; Friedman et al. 2008). Details on the molecular processes that cause cirrhosis to develop into liver cancer are unidentified. Additionally, hepatocytes that experience periodic necrosis and regeneration as a result of increased cell turnover become more sensitive to the

negative effects of certain mutagenic agents. In conclusion, dysplastic foci, nodules, and ultimately HCC may arise from genetic and epigenetic modification (Severi et al. 2010; Roskams and Kojiro 2010; Friedman et al. 2008). The growth pattern of HCC is characterized by three most prevalent cellular patterns: solid (or compact), pseudo glandular (or pseudoscalar), and trabecular. The same lesion exhibits both of these patterns coexisting. Additionally, the cells have the ability to change patterns suddenly (Quaglian 2018). Tumor cells have been found to exhibit a number of variations, including (a) fat accumulation, (b) disseminated differentiation of cytoplasm (clear cell change), (c) incorporation of Mallory–Denk bodies, and oncogenesis (Ziol et al. 2018; Salomao et al. 2010; Okamura 2005; Si et al. 2004). Another variation is appearance of ground glass. Intrahepatic cholangiocarcinoma (iCCAs) and certain subtypes of hepatic neoplasia, including HCC with CK19-positive cells, have been shown to exhibit stem cell-like characteristics and intra- and/or inter-morphological heterogeneity (Singh et al. 2013; Wang et al. 2011; Lee et al. 2006; Roskams et al. 2003).

Cancer stem cells and their significance in HCC

Single or a small number of cells undergo transformation and ongoing growth throughout the early stages of tumor development, starting as a subpopulation of altered cells that direct the growth and progression of the tumor (Merlo et al. 2006). According to this theory, which is referred to as the "stochastic or clonal evolution model," a single mutant cell has the capacity to proliferate unchecked, which can lead to tumor formation and resistance development (Aravalli et al. 2013). The idea emphasizing the function of cancer stem cells (CSCs) in developing HCC is sufficiently supported by the fact that neoplastic hepatocytes like Huh-7 and PLC-5 contain a side population (SP) of cells with stem cell-like properties (Chiba et al. 2006). Progenitor cells may be the source of HCC, according to the results of multiple experimental investigations (Sia et al. 2017). In mice, for instance, genetic changes affect the Hippo pathway in the liver, which leads to the growth of progenitor-like cells and, ultimately, the development of mixed hepatocellular cholangiocarcinoma (HCC-CCA), iCCA, and HCC (Lee et al. 2010).

H-Ras and SV40LT, two active oncogenes, can alter various mice hepatic cells, including hepatocytes, hepatoblasts, and hepatic progenitors, resulting in the development of iCCA or HCC (Holczbauer et al. 2013). In peribiliary glands found throughout the biliary system, there

may be a subpopulation of stem cells or progenitor cells that leads to iCCA and fibrolamellar HCC (FLC) (Cardinale et al. 2012; Torbenson 2012; Cardinale et al. 2011).

Moreover, HCC and iCCA grow as a result of the stimulation of the Notch signaling system (Zender et al. 2013; Villanueva et al. 2012). Together with K-Ras (Kirsten rat sarcoma viral oncogene homolog) mutation, iCCA has a significantly higher abundance of mutated genes encoding isocitrate dehydrogenase than HCC. These genes are linked to decreased differentiation of hepatocytes. They promote oval cell cellular proliferation, and cause biliary transformation following liver injury (Ikenoue et al. 2017; Saha et al. 2014). To put it succinctly, the experimental evidence mentioned above gives the required push regarding the potential of progenitor cells in the formation of the two most common primary liver malignancies, iCCA and HCC.

Additionally, a number of experimental findings demonstrated that adult hepatocytes may be a cell of origin for the development of liver cancer (Marquardt 2016; Shin et al. 2016; Jörs et al. 2015; Mu et al. 2015; He et al. 2013). The results of research using the fate-tracing approach have shown that adult hepatocytes, not progenitor cells, are responsible for the development of liver cancer, as seen in the carcinogen-free (Mdr2 deletion) and hepatoxin-induced models (Shin et al. 2016; Mu et al. 2015; Jörs et al. 2015).

Remarkably, the data indicated that progenitor markers as Foxl1+, EPCAM (epithelial cell adhesion molecule), SOX9 (SRY-Box Transcription Factor 9), and PROM1 (prominin 1)-expressing cancer cells did not significantly contribute to the development of HCC tumors (Shin et al. 2016). Furthermore, this study emphasized that HCC did not originate from biliary cells, which is consistent with previous study (Mu et al. 2015). The study claimed that p62 expression in hepatocytes led to c-MYC upregulation, mTORC1 activation, and eventually the start of HCC (Umemura et al. 2016). Adult hepatocytes' increased adaptability enables them to remain consistent with the cells that cause cancer. Therefore, more thorough research is needed to determine the circumstances that cause adult hepatocytes to change and develop tumors. Lastly, the study will help individuals with severe liver impairment bridge the gap between pre-clinical and clinical stages. Cells that become extremely vulnerable to oncogenic mutations linked to HCC, such as CTNNB1 and those in the TERT promoter (Sia et al. 2017).

HCC and inflammation

Inflammation in the liver can be caused by bacteria, viruses, alcohol, drug, and chemical metabolites. Toxic compounds that cause liver damage typically accumulate as a result of the liver's defective hepatic metabolism, which prevents the liver from converting medications and chemicals into non-reactive or non-immunogenic substances (Fallot et al. 2012; Severi et al. 2010). Kupffer cells and other cells release chemokines and cytokines in response to liver injury, which causes inflammation to occur (Singh et al. 2018; Aravalli et al. 2013).

The pathophysiology of liver cancer can also be greatly impacted by unchecked cellular growth and liver inflammation. According to numerous studies (Singh et al. 2018; Aravalli et al. 2013; Naugler and Karin 2008a), the host immune response triggered by the viral antigen is the primary cause of liver damage caused by viral hepatitis. Hepatocytes have cell surface receptors for cytokines, namely TNF-α, IL-1β, and IL-6. Cytokines are both produced by and targeted by liver sinusoidal endothelial cells. Most of them are produced by Kupffer cells (Leonardi et al. 2012). According to a number of studies (Nakagawa et al. 2009; Wong et al. 2009; Naugler and Karin 2008b), IL-6 is the most significant cytokine among the others because of its direct link with HCC, particularly when cirrhosis is present. Activated Kupffer cells produce more IL-6 when a person has chronic hepatitis. Diethylnitrosamine (DENA)-induced HCC growth was significantly reduced in IL-6 knockout mice, indicating the direct role of IL-6 signaling in experimental carcinogenesis (Aravalli et al. 2013; Singh et al. 2018). IL-6 has been shown to significantly increase the growth of hepatic tumor cells by inhibiting the process of apoptosis, activating signal transducer and activator of transcription 3 (STAT3) (Yu et al. 2009). Additionally, IL-6 creates a crucial connection between obesity and HCC. Gender biases in the development of HCC are indicated by the substantial inhibition of IL-6 production in Kupffer cells by estrogen (Aravalli et al. 2013). The pro-inflammatory immune mediator that Kupffer cells and other immune cells produce is called TNF-α. Prolonged tissue damage alters the NF-κB and Akt pathways and may contribute to the growth and spread of tumors. Additionally, it can create the metabolite 8-oxo-deoxyguanosine (8-oxodG), which causes oxidative stress in primary murine hepatocytes and causes significant DNA damage (Leonardi et al. 2012). Curiously, the function of TNF-α remains uncertain because published research shows that HCC is associated with both increased and decreased TNF-α expression (Leonardi et al. 2012; Aravalli et al. 2007). HSCs are activated, proliferate, and undergo

transdifferentiation into the myofibroblastic phenotype by IL-1β. Additionally, it causes HSC to produce and activate MMP, particularly MMP-9. TNF-based apoptosis-inducing ligand was expressed in neoplastic hepatocytes such HepG2, Hep3B, and Huh-7 in response to IL-1β (Leonardi et al. 2012; Han et al. 2004). The positive stimulus of NF-κβ in a transgenic mouse model was emphasized by the inflammatory HCC model (hepatocyte-specific lymphotoxin αβ transgenic mouse model). By inhibiting NF-κβ, the hepatocyte-specific deletion inhibitor of nuclear factor kappa-B kinase (IKK-β) significantly slowed the progression of HCC (Singh et al. 2018).

Patients with metastatic HCC who are HBV-positive exhibit a Th1/Th2-like cytokine shift worldwide. Th2-like cytokine profile (IL-4, IL-8, IL-10, and IL-5) expressions are significantly elevated as a result of the alteration, while Th1-like cytokine expressions (IL-1α, -1β, IL-2, IL-15, IL-12p40, IL-12p35, TNF-α, and interferon–γ (IFN-γ)) are subsequently decreased. Research has shown that cytokine shift and metastatic phenotype are directly correlated, suggesting that anti-inflammatory and immune-suppressive responses may contribute to the development of HCC (Aravalli et al. 2013; Budhu et al. 2006). Single-nucleotide polymorphisms (SNPs) in different cytokines have been emphasized in an increasing number of discoveries in the literature. One such example is the use of the C31T polymorphism in IL-1β as a genetic marker for hepatitis-related HCC. Similarly, TRAIL 1 receptor polymorphisms C626G and A628C suggested a higher risk of HCC development (Bochud et al. 2012; Joshita et al. 2012, Marabita et al. 2011).

HCC and oxidative stress

By controlling a steady metabolic energy input regulated by enzymes with a wide range of cellular functions, all living systems maintain a reducing cellular redox state within their cells. Oxidative stress results from a disruption in the normal redox state, which raises ROS and RNS. High levels of ROS are produced by persistent oxidative stress in liver cirrhosis and chronic hepatic inflammation, regardless of the cause, according to published research.

In liver, neutrophils, cytochrome P450 enzymes, endotoxin-activated Kupffer cells profoundly generate free radicals (Das et al. 2010). ROS and RNS are crucial for the transcription and activation of growth factors and cytokines, which contribute to the development and spread of

HCC. Under the physiological level, ROS levels are crucial for a number of cellular functions, including senescence, apoptosis, cellular proliferation, and oxidative phosphorylation. Several antioxidant enzymes, including glutathione peroxidase (Gpx), catalase (CAT), thioredoxin (TRX), and superoxide dismutase (SOD), are essential for keeping ROS levels within physiological bounds (Ghosh et al. 2014).

High quantities of ROS are produced as a result of chronic inflammation-induced oxidative stress, and because antioxidant enzymes cannot neutralize them, they assault mitochondria. Because of its restricted genetic repair system, absence of protective histones, and close proximity to a high concentration of ROS, mitochondrial DNA (mtDNA) is vulnerable to attack (Trachootham et al. 2009). Calcium ion homeostasis, oxidative phosphorylation, and other metabolic processes that depend on mitochondria are altered when mtDNA is mutated. A number of neoplastic traits, including unchecked cell proliferation, cellular death signaling pathway disruption, mitogenic signaling activation, epithelial-mesenchymal transition promotion, angiogenesis, metastasis, and chemo-resistance, are acquired by normal hepatocytes as a result of ROS leaking from mitochondria, which causes DNA mutation and physiological and biochemical changes in the cells (Wang et al. 2018).

In vitro and in vivo, malignant cells (Wang et al. 2018; Ma et al. 2016; Dong et al. 2016; Trachootham et al. 2009) are adapted to function at a higher level of endogenous stress than normal cells, according to a substantial number of publications. Furthermore, ROS use MAPK and oxidative alteration to trigger a variety of signaling cascades, including Ras/Raf. Literature reports have identified ROS-induced p38 MAPK activation as one of the major factors causing HCC to advance (Aravalli et al. 2013). GSH/glutathione disulfide (GSSG), MDA, superoxide radicals, and redox enzyme activity were all much higher in HBV-associated HCC patients than in healthy people (Andrea et al. 2015; Marra et al. 2011).

Transition of epithelium to mesenchymal tissue (EMT)

Due of its role in the production of mesoderm, which organizes into numerous layers, EMT is essential during embryogenesis. In cancer, EMT is often activated during metastasis and progression. Additionally, the acquisition of EMT characteristics is closely correlated with the development of chemoresistance and recurrence in cancer. Both the invasive metastatic traits commonly seen in cancer and the stem cell traits can be produced by EMT (Iwatsuki et al.

2010; Thiery et al. 2009). The development of tumors was also significantly influenced by a variety of transcription factors, including Twist, Snail, and Slug, which were active during embryogenesis (Sanker et al. 2019). EMT and tumor growth are stimulated by TGF-β released by stromal liver parenchyma cells and increased PDGF signaling in Ras-transformed malignant hepatocytes. Snail, Slug, Twist, and Vimentin are frequently upregulated in liver cancer, indicating a bad prognosis, while E-cadherin (CDH1) and hepatocyte nuclear factor (HNF)-4α are frequently downregulated (Aravalli et al. 2013). The significance of this four-gene signature in the molecular classification of HCC is highlighted by the poor prognosis shown by CDH1, an inhibitor of DNA binding 2 (ID2), MMP 9, and transcription factor 3 (TCF3) in HCV-infected HCC patients (Kim et al. 2010; Ding et al. 2010).

Hypoxia and HCC

The development of HCC is literally accelerated by hypoxia, including angiogenesis, metastasis, proliferation, and the radio- and chemoresistance of HCC cells. Epithelial-mesenchymal transitions (EMT), intravasation, oxidative defense, extravasation, invasion of the extracellular matrix, and secondary growth of metastases are just a few of the signature features of malignancy that are promoted by hypoxia-inducible factor-1 (HIF-1), an essential oxygen-dependent DNA regulatory element that facilitates the overall development of malignant transformation at a low partial pressure of oxygen (Li et al. 2018).

HIF-1α causes frustration and a bad prognosis in HCC, according to a growing body of evidence in published literature (Yu et al. 2017; Bowyer et al. 2017; Wilson et al. 2014; Thambi et al. 2014; Luo et al. 2014).

CA9 is one of the key tumor markers of HCC because it promotes the expression of cytokines in Hep3B cells under hypoxic conditions. According to a comparative microarray analysis of the gene expressions of HepG2 cells in chronic hypoxia and those in patients with HCC, chronic hypoxia led to the expression of seven sets of different genres, including cyclin D1 and FGF21 (van Malenstein et al. 2010). Additionally, in hypoxic HCC cells, the high-mobility group box (HMGB1) increases the expression of inflammatory cytokines and caspase-1, which causes the tumor to become widely invasive and spread. Chemo-resistance was developed in HCC cells by hypoxia-induced autophagy (Du et al. 2012; Yan et al. 2012; Song et al. 2009).

Cellular signaling pathways involved in HCC

The multi-stage, intricate process of hepatocarciogenesis involves several signaling pathways. The signaling pathways implicated in hepatocarcinogenesis are mentioned here.

EGF/TGF-α pathway
(Scaggiante et al. 2016; Komposch and Sibilia 2016; Buckley et al. 2008).

HGF/c-Met pathway
(Alessandro et al. 2015; Giordano and Columbano 2014; Ang et al. 2013).

Insulin growth factor (IGF) pathway
(Mekuria and Abdhi 2017; Pollak 2012, Breuhahn and Schirmacher 2008).

MAPK pathways
(Yu et al. 2015; Min et al. 2011; Roskoski 2010; Schmitz et al. 2008).

PI3K/Akt/mTOR pathway
(Menon et al. 2012; Bunney and Katan 2010; Llovet and Bruix, 2008; Villanueva et al. 2008; Sabatini 2006).

Signaling pathways involved in neovascularization in HCC
(Elsamanoudy et al. 2016; Nana et al. 2016; Tumanova and Shchegolev 2015).

Pathways related to cell differentiation and development

Wnt-β catenin pathway
(Wasiberg and Saba 2015; Saito-Diaz et al. 2013; Saito-Diaz et al. 2013).

Hedgehog (Hh) signalling
(Lee et al. 2016).

Hippo pathway
(Kang et al. 2016; Hong et al. 2015; Vicente et al. 2015; Su-Xia et al. 2014; Li et al. 2012a).

Notch pathway (Kevin et al. 2015; Yamamoto et al. 2014; Villanueva et al. 2012; Gramantieri et al. 2007).

Target receptor(s) and biomarker proteins in HCC

Asialoglycoprotein receptor
(Elanggar et al. 2019; Quan et al. 2015; Thapa et al. 2015; D'Souza and Devarajan 2015; Trere et al. 1999; Hyodo et al. 1993; Weigel 1982; Weigel 1981).

Glypican-3 (GPC3)
(Zhang et al. 2016; Wang et al. 2016; Hanaoka et al. 2015; Mu et al. 2014; Feng et al. 2013; Polakis 2012; Li et al. 2012b; Capurro et al. 2005; Filmus and Selleck 2001).

Golgi protein73 (GP73)
(Block et al. 2010; Norton et al. 2008; Kladney et al. 2000).

Heat shock protein 70 (HSP70)
(Jin et al. 2011; Fang et al. 2012; Liu et al. 2014).

Homodimeric glycoprotein (AF-20)
(Zhang et al. 2016; Moradpour et al. 1995; Wilson et al. 1988).

Transferrin receptor (TfR)
(Tros de Ilarduya and Duzgunes 2013; Tortorella and Karagiannis 2014).

Tumor-associated glycoprotein-72 (TAG-72)
(Chauhan and Lahiri 2016; Zhang et al. 2012; Chauhan et al. 2007).

Ki-67 antigen
(Scholzen and Gerdes 2000; Mohamed et al. 2008)

Somatostatin receptor (SSTR)
(Reynaert et al. 2004; Blaker 2004; Notas 2004; Kouroumalis et al. 1998).

Osteopontin (OPN)
(De Stefano et al. 2018; Duarte-Salles et al. 2016; Fouad et al. 2015; Liu et al. 2015; Qin 2014, Wang et al. 2014; Shang et al. 2012; Weber 2011).

Squamous cell carcinoma antigen (SCCA)
(Lou et al. 2017; Pozzan et al. 2014).

fin 1 (HepPar1)
(Villari et al. 2002; Zimmerman et al. 2001; Xue et al. 2015).

Epidermal growth factor (EGFR)
(Hu et al. 2015; Berasain and Avila 2014; Lanaya et al. 2014).

Hepatocyte growth factor (HGF)
(Mizuguchi et al. 2004; Yamagamim et al. 2002; Nakamura et al. 1997).

α-1-Fucosidase
(Wang et al. 2014; Tangkijvanich et al. 1999).

Annexin A2
(Zhang et al. 2015; Lokman et al. 2014; Sun et al. 2013).

APO-J
(Zheng et al. 2015; Nafee et al. 2012; Comunale et al. 2011).

DKK-1 (Dickkopf-p1)
(Shen et al. 2016; Zhu et al. 2013; Tsai et al. 2012).

Human carbonyl reductase 2 (HCR2)
(Wang et al. 2009; Liu et al. 2006).

Midkine
(Nault 2014; Zhu et al. 2013).

Nerve growth factor (NGF)
(Rasi G et al. 2007; Levi-Montalcini 1987).

Vascular endothelial growth factor (VEGF)
(Zhu et al. 2013; Llovet et al. 2012; Poon et al. 2004; Treiber et al. 2006).

Transforming growth factor-β (TGF-β)
(De Stefano et al. 2018; Lou et al. 2017; Li et al. 2015; Bedossa et al. 1995).

Micro RNA
(Jin et al. 2019; De Stefano et al. 2018; Xiong et al. 2018; Jin et al. 2017; Lou et al. 2017; Shen et al. 2016; Li et al. 2016; Chauhan and Lahiri 2016; Bertoli et al. 2015; Jiang et al. 2015; Lin et al. 2015; Casanova-Salas 2014; Su et al. 2014; Zhu et al. 2014; Liang et al. 2013; Zhang et al. 2013; Janssen et al. 2013; Macfarlane et al. 2010; Yang et al. 2010; Li et al. 2010; Huang et al. 2009).

Circular RNAs
(Han et al. 2018; De Stefano et al. 2018; Han et al. 2017; Yao et al. 2017)

Cancer stem markers
(Chen et al. 2016; Chen et al. 2013b; Reya et al. 2001).

CD44

(Xie et al. 2008; Sano et al. 2003; Endo and Terada 2000).

CD133

(Yin et al. 2007).

CD90

(Sukowati et al. 2013; Yamashita et al. 2013; Yang et al. 2008).

Therapeutic interventions

Due to the lengthy latent period linked to HCC, individuals are diagnosed with the disease at an advanced stage, when they exhibit symptoms and some degree of liver impairment. At this point, no treatment plan offers survival. Furthermore, all of the existing therapies have been linked to significantly elevated morbidity. One-fourth of the patients with HCC were unaware of their condition before to its discovery, according to a study evaluating the Marketscan database of 700,000 patients who had to apply at least once for NAFLD/NASH/HCV.

According to the study, routine HCC screening was performed in 21.1% and 22.3% of individuals with HCV infection. It's interesting to note that a third of patients who were continuously monitored by knowledgeable hepatologists had irregular HCC screening. Early diagnosis and restoration of liver function may be achieved with appropriate screening and attentive monitoring (Yang et al. 2019).

Resection through surgery

Among the different treatment options for noncirrhotic individuals, surgical resection has the most promise and offers a high cure rate with a 5-year survival rate ranging from 41% to 74%. The faith of the resection is determined by a number of parameters, including the tumor's size and position, basal liver function, the condition of the residual (non-tumor) liver volume, and a tumor-free area of the liver appropriate for post-resection mortality and morbidity. There is only one main tumor in the liver and no radiologic evidence of vascular invasion in the typical resection candidates. Liver function can be sufficiently maintained by the remainder of the liver. Choosing the best candidates is crucial to lowering the risk of surgery-related death and

morbidity. Candidates with a proper synthetic function and sufficient residual volume (volume of the liver without tumor) preserve the technical element of operation. While partial hepatectomy is favored in cases of cirrhosis or benign liver tumors, major hepatectomy is necessary for the removal of big tumors. Additionally, cirrhotic patients with numerous tumors and extra-hepatic masses, central bile duct involvement, and portal hypertension are not surgically resected (Yang et al. 2019; Marrero et al. 2018; Balogh et al. 2016; Yang et al. 2012).

Surgery is advised for patients with advanced HCC, which is defined as large tumors larger than 10 cm in diameter, extrahepatic metastases, or macroscopic vascular invasion, when no other treatment option, such as chemotherapy, liver transplantation, or systemic ablation, is effective.

Between 40 and 166 patients with significant HCC (diameter greater than 10 cm) were assessed, and extensive hepatic resection was performed. They were compared to patients with smaller HCC in terms of postoperative mortality, morbidity, and increasing blood loss. Five-year survival rates were 28–33% and mortality rates were 2-3.3% (Kishi et al. 2011). When compared to HCC patients with portal venous invasion of secondary tributaries (Vp2) or peripheral tributaries (Vp1), surgical resection is not advised for patients with HCC who have portal venous invasion of the main trunk (Vp4) or right or left main trunk (Vp3) because of the poor survival benefits (Kishi et al. 2011). On the other hand, reports of aggressive resection for HCC patients with Vp3 or Vp4 indicate 5-year survival rates from 10-42%. Resection of portal vein tumors is accomplished in two steps: first, a particular segment of the portal vein is cut off, and then the tumor bulk is detached and removed from the inner part of the portal vein using the peeling-off technique (Wu et al. 2000). The benefits of aggressive resection in cases of extrahepatic metastases are not very great. According to Yang et al. (2007), the 5-year survival rate for 20 patients who had extrahepatic recurrence following hepatic resection was just 30%.

Radiation therapy

Radiotherapy, sometimes referred to as radiation therapy, is a potent treatment method that uses high radiation doses to reduce tumors and eradicate cancer. High-energy waves or particles, such as protons, gamma rays, electron beams, or X-rays, are used in radiotherapy to

destroy or harm cancer cells. The main way by which radiation kills cancer cells is by causing little DNA fragments to form inside the cells. Cancer cells die as a result of these DNA splits, which also stop them from growing and dividing. Approximately 50% of patients receive radiation therapy at some stage of their cancer treatment.

Both intratumoral and transarterial approaches have been used to investigate the potential of the chitosan complex (Ho199). Transarterial delivery of the 166Ho-chitosan complex was found to be highly effective in treating HCC in another phase, particularly for individuals with tumors measuring 3-5 cm (Sohn et al. 2009). Complete tumor necrosis (tumors < 2 cm) was demonstrated by 91.7% of respondents to a percutaneous 166Ho-chitosan injection in a phase IIb clinical trial, according to Kim et al. (2006).

With a half-life of eight days, iodine 131 (I131) is mostly a beta emitter with a trace amount of gamma emission potential. The medication I131-Lipiodol has been used (Boucher et al. 2007). Lintia-Gaultier et al. (2013) reported that it was also effective in treating non-operable HCC. After receiving 131I-Lipiodol intraarterially, 17–92% of patients experienced a clinical response (Lambert et al. 2005). The conjugates that are 90Y and 188Re labeled show promise, making them a new radiotherapy modality. Microspheres labeled with 90Y are fairly similar to those labeled with radiolabelled lipiodol (Lambert et al. 2005).

Conformal radiotherapy in three dimensions (3DCRT)

Three-dimensional conformal radiation, or 3DCRT, is a cutting-edge imaging method that creates three-dimensional pictures of malignancies as well as the patient's surrounding organs and tissues. Radiation therapy shows great promise for more accurately and efficiently delivering radical tumor doses to a well-defined liver lesion. For individuals with cirrhotic HCC, 3DCRT has been investigated as a potential alternative treatment. On the other hand, it shows little promise for other curative methods such radiofrequency ablation, liver transplantation, and surgical tumor removal.

According to Robertson et al. patients with unresectable, nondiffuse primary hepatobiliary malignancies had a higher chance of survival when they received fluorodeoxyuridine (FdUrd) through the hepatic artery in conjunction with conformal radiotherapy (RT) (Robertson et al.

1997). According to Ben-josef et al. (2005), individuals with intrahepatic malignancies that were not candidates for surgical resection had a longer survival rate when they received high dose targeted liver irradiation (60.75 Gy, 1.5-Gy fraction bid) through the hepatic artery using floxuridine. In a phase II research, Mornex et al. (2006) found that high dose 3DCRT (66 Gy, 2 Gy/fraction) was very effective in treating cirrhosis patients (small HCC, one nodule < or = 5 cm, or two nodules < or = 3 cm), with encouraging outcomes. Numerous benefits, including the ability to treat multiple lesions exclusively in a non-invasive manner, the worldwide accessibility of three-dimensional conformal radiation, and numerous encouraging outcomes, have been widely documented in the literature (Kouloulias et al. 2013).

TARE, or transarterial radioembolization

Similar patients to those treated with transarterial chemoembolization (TACE) are treated with TARE. This method uses lipiodol tagged with iodine or rhenium or a microsphere laden with yttrium (Y90) to be delivered intra-arterially (Sacco et al. 2016). SIR spheres made of resin and thermospheres made of glass at the moment, the two main kinds of microspheres utilized in TARE are thermospheres made of glass and SIR spheres made of resin. Their embolic powers and activities vary greatly. Because TARE can eliminate hepatic ischemia when local radiation is administered to liver tumors, it is recommended for HCC patients with portal vein thrombosis (Cho et al. 2016). This approach implants specific radiation sources inside the tumor, preventing normal liver cells from being exposed to the harmful effects of radiation (Kim 2017). To assess this method, numerous clinical trials were carried out. There are very few trials that show radioembolization is better than TACE in terms of response and adverse effects (Ricke et al. 2015; Vilgrain et al. 2014).

Systemic treatments for HCC

Chemotherapy is the sole therapeutic option accessible for HCC patients because of the lengthy latent period that causes the disease to be initially diagnosed in intermediate and severe stages. When traditional chemotherapy medicines including gemcitabine, doxorubicin, 5-fluorouracil, and cisplatin are administered, a little increase in survival is seen. Additionally, some randomized trials showed that administering the combination chemotherapy improved the

response rate somewhat. The objective reaction, however, remained unchanged (Mohamed et al. 2017; Yeo et al. 2005; Leung et al. 1999).

Conventional chemotherapeutic drugs have a poor treatment outcome and potentially substantial side effects due to a number of causes. Multi-drug resistance in HCC tumors, a quicker rate of clearance, poor drug availability in HCC cells (only 5–10% of the dose accumulated in normal organs), and non-specific distribution are some of the crucial factors (Zhang et al. 2016; Lo et al. 2008; Han and Park 2008).

Therapy in the first line

The medication sorafenib

As the first multi-kinase inhibitor, sorafenib (Nexavar®, Bayer Pharmaceuticals, Berlin, Germany) has been approved by the US Food and Drug Administration (USFDA) as a first-line treatment for advanced HCC. After binding with different extracellular tyrosine kinases, including vascular endothelial growth factor receptors (VEGFR 2, VEGFR-3, VEGFR1), platelet-derived growth factor receptor β (PDGFR β), and downstream intracellular signaling molecules Raf kinases (B-Raf and C-Raf) and serine/threonine kinases (Al-Rajabi et al. 2015; Fitzmoris et al. 2015), it inhibits angiogenesis, cellular proliferation, and metastasis.

According to the findings of the Asia-Pacific research in 2008 and the Sorafenib Hepatocellular Carcinoma Randomized Trial (SHARP) in 2007, sorafenib improved the median overall survival (OS) of patients with advanced HCC (BCLC stage C) by 2.8 months (Keating and Santoro 2009).

Sunitinib (2015), Brivanib (2013), Sorafenib plus Erlotinib (2015), and Linifanib (2015) were among the phase III trials that failed in the field of MKIs (Zhu et al. 2015; Cheng et al. 2013d; Johnson et al. 2013). The two other promising oral MKIs that emerged from the thorough search for MKIs are regorafenib and lenvatinib, which shown encouraging outcomes in clinical trials in 2017 and 2018, respectively (Kim and Abou-Alfa 2014).

Lenvatinib

The USFDA has authorized lenvatinib, a broad-spectrum oral MKI, as a first-line medication for treating advanced HCC. VEFGR 1-3, fibroblast growth factor 1-4 (FGFR1-4), PDGFR-α, c-Kit, and rearranged during transfection (RET) are among the tyrosine kinases that it targets. (Kudo et al. 2018) The USFDA approved lenvatinib based on findings from the randomized open-label, global non-inferiority phase III REFLECT study (Kudo et al. 2018b) in patients with untreated, metastatic, or incurable HCC.

Second-line treatment

Regorafenib is a multi-target tyrosine kinase inhibitor.

For patients with advanced HCC who show signs of sorafenib resistance, the US Food and Drug Administration (USFDA) has suggested regorafenib as a second-line treatment. The primary mechanism of action of regorafenib is its capacity to inhibit a variety of targets, including PDGFR, fibroblast-derived growth factor receptor (FDGFR), colony-stimulating factor 1 receptor (CSF1R), tyrosine kinase and immunoglobin, and epidermal growth factor (EGF) homology domains-2 (TIE-2), and VEGFR1. It functions and is structurally similar to sorafenib (Martin et al. 2008). Additionally, individuals who received sorafenib and a placebo had a shorter survival time (19.2 months) than those who received regorafenib and sorafenib (26 months) (Frennette 2017; Cheng et al. 2017).

Cabozanib

One oral multiple tyrosine kinase inhibitor, cabotezantinib, inhibits oncogenesis, metastasis, and angiogenesis (Yang et al. 2019; Liu et al. 2019). The receptors that cabotinib targets are c-Met, AXL, and VEGFR (1-3) (Huang et al. 2020). In advanced HCC patoents, who had earlier received sorafenib treatment, the results of the phase III CELESTIAL study (Abou-Alfa et al. 2018) showed that cabozantinib treatment significantly improved OS when compared to placebo.

Ramucirumab

A recombinant IgG1 monoclonal antibody called Ramucirumab shows promise as a VEGFR2 antagonist. It suppresses the activation of the VEGF receptor by preventing ligands including VEGFA, VEGFC, and VEGFD from attaching to the receptor. According to Yang et al. (2019) and Liu et al. (2019), ramucirumab was first authorized for the treatment of colorectal cancer on April 29, 2015, non-small cell lung cancer on December 12, 2014, and gastric cancer on April 21, 2014.

Checkpoint Inhibitors for the Immune System

The introduction of multi-kinase inhibitors (MKIs) as chemotherapeutic agents raised high expectations for improved survival rates and treatment outcomes in patients with advanced hepatocellular carcinoma (HCC). However, the results were not as favorable as anticipated. Objective response rates (ORR) and OS did not show significant improvements when MKIs were administered either alone or in combination. Patients receiving MKIs in phase III clinical trials experienced significant drug-related adverse events (AEs), with grade 3 adverse events occurring in 45–70% of cases, leading to dose reductions or discontinuation of treatment. Common AEs included hand-foot syndrome (HFS), diarrhea, hypertension, fatigue, nausea, and vomiting. Moreover, there have been reports of adaptive and acquired resistance to MKIs in HCC patients (Zhu et al. 2017; Rotow and Bivana 2017).

Cancer cells are capable of evading immune surveillance within the tumor microenvironment through various mechanisms of immune suppression. To develop targeted therapies, research is intensively focusing on the immune checkpoint mechanisms and associated molecules (Keenan et al. 2019; Pardee and Butterfield 2012). Monoclonal antibodies targeting these immune checkpoint ligands are being evaluated for their efficacy in treating various cancers, including HCC (Kudo 2018b; Gong et al. 2018). In preclinical liver cancer models, treatment with sorafenib was shown to reduce the presence of regulatory T-cells (T-regs) and myeloid-derived suppressor cells (MDSCs) in the spleen, bone marrow, and tumors, thus impairing their immunosuppressive function (Kudo, 2018b). However, sorafenib also induces hypoxia in the tumor microenvironment by upregulating HIF-α and increasing immune checkpoint markers such as PD-L1. As a result, combining MKIs with immune checkpoint inhibitors may enhance therapeutic outcomes for HCC. Several of these combination therapies are currently

undergoing clinical trials (Wu et al. 2019; Kudo, 2018b; Gong et al. 2018). Nonetheless, challenges remain, particularly concerning immune-related adverse events and patient variability in responses (Kudo 2018b; Gong et al. 2018).

Recent advancements in HCC treatment have been significant, with ongoing clinical trials of drugs like dovitinib, oratinib, vandetanib, and linifanib showing promise for improved survival rates. Inhibiting immune checkpoints related to tumor malignancy has bolstered hopes for HCC therapy. Studies have also indicated that additional branched-chain amino acids (BCAAs) and nutritional supplements can reduce neoplastic activity in HCC patients. Despite the development of new therapies, sorafenib remains the most promising option currently available. Continued research into new drugs, along with the use of site-specific nanoformulations, gene therapy, and antisense therapies, may lead to innovative approaches in cancer treatment.

Antisense Therapy: Design and Delivery for Target-Specific Gene Silencing

Antisense oligonucleotides (ASOs) are short, single-stranded nucleic acids (typically fewer than 50 base pairs) that can bind to a target gene or mRNA through Watson-Crick base pairing, leading to the inhibition or downregulation of gene expression. The potential of ASOs as therapeutic agents was first recognized by Zemencnik and Stephenson (Fattal and Bochot, 2009), who observed that oligonucleotides complementary to the Rous sarcoma virus's 3' end could prevent viral replication in chicken fibroblasts. The specificity of ASOs is based on the unique occurrence of a specific DNA sequence in the human genome (Martimprey et al. 2009), and they have since gained attention for their potential to treat genetic disorders, including cancer (Fattal and Bochot 2009).

The discovery of small interfering RNA (siRNA) technology (21–23 base pair double-stranded RNA molecules) further advanced oligonucleotide-based therapies. The siRNA mechanism involves the enzyme Dicer, which cleaves long double-stranded RNA into smaller fragments. These fragments are then introduced to the RNA-induced silencing complex (RISC), which provides one strand to bind and degrade target mRNA (Dicer et al. 2001). This process is central to RNA interference and is similar to the mechanism of antisense oligonucleotides, where the single-stranded RNA hybridizes with target mRNA, causing cleavage by ribonucleases (RNA interference).

In addition to gene silencing, ASOs can also influence RNA processing events such as splicing, polyadenylation, and the insertion of the 5' cap. ASOs designed to target specific splicing sites

or regulatory regions of mRNA can "switch" protein functions in a reversible manner. For example, oligonucleotides targeting the *Bcl-x* gene have been shown to switch between pro-apoptotic and anti-apoptotic forms, sensitizing cells to chemotherapy (Dias and Stein, 2002). In some cases, ASOs may also alter polyadenylation patterns, redirecting RNA processing to use alternative polyadenylation sites, which can further regulate gene expression.

Despite their promise, the delivery of ASOs into target cells remains a significant challenge. While mechanisms such as fluid-phase pinocytosis and adsorptive endocytosis have been proposed (De Diesbach et al. 2000), ASOs are typically poorly internalized by cells. The liver and kidneys are primary sites for ASO accumulation, with smaller amounts in the spleen, lymph nodes, and bone marrow. Targeting efficient delivery systems is essential for maximizing the therapeutic potential of ASOs.

Antisense oligonucleotide therapy has made substantial progress, but many challenges remain, particularly concerning the optimization of delivery methods and overcoming cellular barriers. Despite over three decades of research, only two ASOs have been FDA-approved (Juliano et al. 2008), highlighting the complexity of translating laboratory findings into clinical success.

Antisense Oligonucleotide Design: Enhancing Efficacy

The development of antisense oligonucleotides has progressed steadily, with various chemical modifications to improve their stability and effectiveness. The most common mechanism of action for ASOs is to hybridize with a target mRNA sequence, forming a heteroduplex that leads to cleavage by RNase H enzymes, thereby preventing translation of the target gene. This process is highly effective, as the RNase H enzyme selectively cleaves the RNA strand in the heteroduplex, allowing the ASO to remain intact and available for further targeting (Caffo et al. 2013; Pirollo et al. 2003). This RNase H-dependent mechanism is widely utilized in antisense therapy, but other methods, such as steric blocking of translation initiation, also contribute to gene silencing (Behlke et al. 2005).

For RNase H-dependent ASOs to work effectively, they must bind to their target mRNA with high specificity. The binding affinity is influenced by factors such as the GC content of the ASO and the thermodynamic stability of the duplex. Modifications to the ASO structure, such as phosphorothioate backbones and 2′-fluoro modifications, have been developed to enhance their nuclease resistance while maintaining their ability to induce RNase H activity (Damha et al. 2001).

Despite these advances, challenges remain, particularly with partial hybridization and off-target effects. Chimeric oligonucleotides have been explored to minimize these issues, but further research is needed to optimize their design for clinical applications (Monia et al. 1993; Giles et al. 1998).

Ultimately, the design of antisense oligonucleotides is a highly tailored process that depends on the specific biological targets and the therapeutic objectives. As research continues, improved designs and delivery systems will likely expand the range of diseases treatable with ASOs.

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Therefore, lines that guarantee the target sequences' accessibility inside the folded RNA structure must be used while building an ASO for the aforementioned pathway.

Antisense Mechanisms Independent of RNase H

Antisense oligonucleotides (ASOs) can exert their effects through RNase H-independent pathways by interfering directly with the translation process. These mechanisms involve physically hindering ribosome assembly, blocking the translation initiation codon, or preventing the elongation of the nascent polypeptide chain (Dias and Stein 2002; Baker et al. 1997). For example, a phosphodiester oligonucleotide relying on RNase H activity generated a truncated protein product of the same size as one produced by a peptide nucleic acid (PNA) that acted on H-Ras mRNA through a steric-blocking mechanism (Dias et al. 1999).

In cases where mRNA secondary structure does not necessitate unwinding, certain RNA regions that typically interact with ribosomes, spliceosomes, or other cellular components may remain accessible to ASOs. Such sequences are promising therapeutic targets (Behlke et al. 2005). Initial studies focused on targeting the mRNA cap structure, translation start site (AUG), and the 3′-end (Goodchild et al. 1988). While more than 70% of ASOs have historically targeted the AUG start codon (Dias and Stein 2002), this region is not always the optimal binding site. Untranslated regions (UTRs), particularly the 5′ and 3′ ends, have also proven to be suitable alternatives (Mologni et al. 1998; Skorski et al. 1997).

Blocking the core coding sequence has generally shown lower efficacy compared to targeting UTRs or the AUG region (Dias and Stein, 2002; Tyler et al. 1999; Gryaznov et al. 1996; Monia et al. 1993), likely due to the ability of ribosomes to displace ASOs prior to translation initiation. Another RNase H-independent mechanism involves altering pre-mRNA splicing, which can lead to the production of nonfunctional transcripts that are degraded via nonsense-

mediated decay (Aartsma-Rus et al. 2005; Kole et al. 2004). This approach also allows for the correction of aberrant splicing or modulation of alternative splicing patterns.

Splice-modifying ASOs must enter the nucleus, where they bind to pre-mRNA. These oligonucleotides typically target splice donor and acceptor sites, or branch point sequences crucial for exon definition. Compared to splice site-targeting ASOs, those aimed at exonic splicing enhancers (ESEs) offer broader flexibility since ESEs are variable and not constrained by strict consensus sequences (Aartsma-Rus et al. 2005).

ASO efficacy is also influenced by factors such as oligonucleotide length, nucleotide composition, and the thermodynamic properties of the ASO-mRNA duplex (Chan et al. 2006). Successful hybridization requires that the duplex's free energy is lower than the sum of the free energies of the unbound ASO and target RNA (Mathews et al. 2004). Effective binding also depends on the presence of single-stranded regions in both the ASO and target mRNA. Since ASOs are typically short (15–25 nucleotides), they rarely form stable secondary structures themselves but can form dimers or complexes with similar ASOs. These interactions, including ASO-target and ASO-ASO binding affinities, can be predicted computationally (Mathews et al. 2004).

Designing ASOs for translation inhibition often involves in silico tools that consider the start codon region as a key target. However, despite advances in predictive algorithms, the development of effective ASOs often still requires empirical testing (Aartsma-Rus et al. 2005). Certain backbone chemistries, such as 2′-O-alkyl groups, peptide nucleic acids, and morpholinos, enhance splicing modulation by resisting degradation and improving binding affinity (Kole and Sazani, 2001). These RNase H-inactive analogs, particularly morpholinos and 2′-O-alkyl-modified ASOs, are widely used for their strong hybridization capacity, which effectively prevents the bound RNA from participating in splicing or translation (Arora et al. 2000; Iversen, 2001; Mercatante et al. 2001).

Structural Considerations in ASO Design

The secondary and tertiary structures of mRNA can significantly influence ASO binding efficiency, as certain regions may become structurally concealed and inaccessible. Therefore, to enhance the likelihood of successful hybridization, it is essential to identify and target mRNA regions that are more likely to remain single-stranded (Aartsma-Rus et al. 2005; Ding 2001). Accurate prediction of mRNA folding patterns plays a pivotal role in guiding effective ASO design (Chan et al. 2006; Andronescu et al. 2005).

To this end, various computational tools have been developed to model RNA secondary structures and evaluate their accessibility. One commonly used metric is the "single-strand (SS) count," which reflects how often a nucleotide remains unpaired across a range of predicted conformations (Aartsma-Rus et al. 2008). ASOs generally perform better when designed against these consistently single-stranded regions. This probabilistic approach often provides a more realistic prediction of in vivo ASO efficacy compared to models that consider only the most thermodynamically stable RNA structure (Aartsma-Rus et al. 2008).

Earlier work by Matveeva et al. (1997) emphasized the challenges of locating reliable binding sites solely through structural predictions, noting that randomly selected regions only occasionally yielded highly effective targets. Nevertheless, tools like Foldsplit and newer programs have since improved ASO screening capabilities (Behlke et al. 2005; Patzel et al. 1999). Among widely used platforms, Mfold (http://www.bioinfo.rpi.edu/applications/mfold) provides estimations of minimum free energy (ΔG) for various RNA conformations, including both optimal and suboptimal structures (Zuker, 2003; Chan et al. 2006; Aartsma-Rus et al. 2008). Sfold (http://sfold.wardsworth.org/index.pl), another popular tool, focuses on predicting the most probable single structure of a given RNA sequence (Ding and Lawrence, 2001). Integrating both approaches is often necessary to obtain a comprehensive overview for rational ASO design.

Interestingly, regions predicted to be single-stranded are not always the most effective targets. In some studies, potential ASO binding sites were located in structured regions, while many predicted accessible regions were unsuitable (Behlke et al. 2005). Although Watson-Crick base pairing is fundamental to hybridization, additional molecular factors can interfere. For instance, regions appearing accessible might be involved in stacking interactions, hydrogen bonding, or hydration shells that impede ASO binding. While ASOs can disrupt these structures, the resulting conformational changes may destabilize the overall RNA structure and reduce binding efficiency (Behlke et al. 2005).

Even minor alterations in ASO length or a shift of one or two nucleotides can significantly affect hybridization kinetics (Sczakiel 2000; Kronenwett et al. 1996; Rittner et al. 1993). Some unpaired RNA regions may also adopt helical conformations that favor ASO binding. Structural motifs such as terminal loops, bulges, internal loops, junctions, and hairpins—especially those with ten or more consecutive nucleotides—are often the most accessible to ASOs (Kretschmer-Kazemi and Sczakiel 2001).

A particularly stable RNA-RNA interaction, known as a "kissing complex," involves loop-loop interactions between two hairpins and can influence natural antisense regulation (Behlke et al. 2005). Additionally, nucleotide composition and structural positioning—such as preferential binding to the 5′ side of loops—can affect hybridization behavior (Puri and Chattopadhyaya 1999; Bruice and Lima 1997).

To streamline target identification, a tool called Target-Finder

(http://www.bioit.org.cn/ao/targetfinder.com), based on the mRNA Accessible Site Tagging (MAST) approach, was developed to locate prospective ASO binding sites (Bo and Wang 2005). Highly conserved sequence motifs have been identified as strong ASO targets (Yang et al. 2003). Furthermore, evaluating conserved structural elements across multiple optimal and suboptimal predicted RNA conformations can enhance the likelihood of selecting effective ASO binding regions (Chan et al. 2006).

ASO elements

Influence of ASO Length on Hybrid Stability

The length of antisense oligonucleotides (ASOs) plays a significant role in determining the stability and specificity of ASO–mRNA interactions. Short oligonucleotides are generally more specific, as they are less likely to encounter unintended partially complementary sequences. In contrast, longer ASOs have a higher probability of hybridizing with off-target transcripts due to partial complementarity. This can still result in RNase H-mediated cleavage, as only a short stretch—approximately five consecutive base pairs—is sufficient to activate the enzyme (Behlke et al. 2005). Based on these factors, an optimal ASO length is typically around 20 nucleotides. This length strikes a balance between specificity, efficiency, and ease of chemical synthesis. Additionally, 19-base sequences are statistically unique in the human genome, minimizing the risk of unintended hybridization (Jiménez and Durbin 2006). However, in certain cases where the RNA structure imposes constraints, shorter ASOs may still be effective (Wagner et al. 1996; Disney et al. 2000). Nevertheless, most studies report a significant reduction in efficacy when ASO length is decreased to around 10 bases or fewer (Cowsert et al. 1993).

Sequence Composition and Functional Elements

Optimizing length alone is insufficient without evaluating the ASO sequence for potential motifs or features that could affect functionality. Sequence elements that promote self-complementarity or unintended binding to non-target RNAs can hinder hybridization and reduce activity. Certain motifs have been correlated with decreased ASO performance, such as GGGG (which can form G-quartets), ACTG, AAA, and TAA. Conversely, other sequences, like CCAC, TCCC, ACTC, GCCA, and CTCT, are positively associated with efficacy (Matveeva et al. 2000; Chan et al. 2006; Behlke et al. 2005).

Cytosine–guanine (CG) dinucleotide content is another critical feature to consider. Unmethylated CG motifs, prevalent in bacterial DNA, are recognized by the human immune system as potential pathogen-associated molecular patterns. Toll-like receptor 9 (TLR9) on immune cells can detect these motifs, leading to immune activation, including cytokine release, B-cell proliferation, and stimulation of various immune cell types (Vollmer et al. 2004; Krieg et al. 1999). The immunostimulatory potential of CG sequences is influenced by their flanking nucleotides, with the purine-purine-CG-pyrimidine-pyrimidine motif being particularly potent (Krieg and Stein, 1995). These immune responses may contribute to side effects such as splenomegaly, lymphoid hyperplasia, or systemic inflammation (Levin, 1999). One strategy to avoid these issues is to exclude CG-containing motifs, especially those flanked by immunostimulatory sequences, or to incorporate 5-methylcytidine in place of cytosine to reduce immunogenicity without compromising hybridization efficiency (Behlke et al. 2005).

Another structural concern involves the potential formation of higher-order structures such as G-quadruplexes or tetraplexes. These can form in G-rich sequences containing motifs like GG, GGG, or GGGG and can interfere with ASO activity or induce off-target protein interactions (Williamson et al. 1989; Schultze et al. 1994). Some G-rich ASOs may bind to proteins such as thrombin (Griffin et al. 1993), the HIV envelope protein (Wyatt et al. 1994), or transcription factors (Tam et al. 1999), resulting in unintended biological effects. The likelihood of forming such structures can be reduced by substituting modified nucleotides like 7-deazaG or 6-thioG in place of guanine (Murchie and Lilley 1994; Olivas and Maher 1995). Tetraplex formation has also been observed in cytosine-rich phosphorothioate oligonucleotides (Wang et al. 1998). For these reasons, sequences with long homopolymeric runs or multiple consecutive identical nucleotides should be treated cautiously during ASO design, even if not all such sequences necessarily form higher-order structures under physiological conditions.

Thermodynamic Factors in ASO Design

Thermodynamic stability is another crucial parameter in antisense oligonucleotide development. The interaction energies between an ASO and its target mRNA—as well as between multiple ASO molecules—can be predicted using tools like OligoWalk (http://128.151.176.70/RNAstructure.html) part of the RNA structure 3.5 software suite (Mathews et al. 1999, 2004). Ideally, the free energy of hybridization between the ASO and target RNA should be sufficiently negative ($\Delta G_{037} < -8$ kcal/mol) to ensure a stable duplex, while the energy for ASO–ASO dimer formation should be comparatively less favorable ($\Delta G_{037} \geq -1.1$ kcal/mol), minimizing self-interaction (Matveeva et al. 2000). Data from antisense databases at ISIS Pharmaceuticals further support that meeting these criteria substantially increases the probability of successful gene silencing (Matveeva et al. 2003).

For example, using these parameters, ASOs targeting vascular endothelial growth factor (VEGF) have demonstrated suppression efficiencies as high as 85% (Fei and Zhang 2005). However, applications targeting pre-mRNA splicing—such as in the case of Duchenne muscular dystrophy (DMD)—often deviate from these thermodynamic norms. Splicing occurs in the nucleus and targets pre-mRNA, rather than mature cytoplasmic mRNA, as is the case with RNase H-mediated pathways (Aartsma-Rus et al. 2008). In these nuclear applications, ASO–ASO interactions may enhance nuclear retention and reduce nonspecific interactions, thus improving efficacy.

To account for the complexity of these parameters, advanced predictive models like the AOpredict algorithm (http://www.cgb.ki.se/AOpredict) use neural networks to evaluate numerous features, including sequence content, RNA–ASO binding thermodynamics, terminal base composition, and known efficacy-related motifs (Chan et al. 2006). This model reportedly predicts ASOs with over 50% knockdown efficiency with 92% accuracy. However, its stringency may lead to the exclusion of functionally effective sequences, and it occasionally misinterprets the thermodynamic implications of dimerization. Consequently, theoretical design should be complemented by empirical validation through systematic screening to identify the most effective ASO candidates.

Chemistry of Oligonucleotides

Despite their ease of synthesis, phosphodiester oligonucleotides are limited in therapeutic applications due to rapid degradation by cellular endo- and exonucleases—primarily acting in a 3′ to 5′ direction—and their highly charged nature, which hampers cellular uptake (Chan et al. 2006; Dias and Stein 2002). Moreover, the breakdown products—mononucleotides like

dNMP2—generated through ecto-5′-nucleotidase activity on the cell surface, can exert cytotoxic and antiproliferative effects by interfering with key enzymes such as thymidine kinase (Koziolkiewicz et al. 2001; Vaerman et al. 1997). Modifications to oligonucleotides have been found to improve their intracellular half-life and preserve their ability to recruit RNase H for RNA target degradation (Behlke et al. 2005). Over time, several chemical modifications have been introduced to enhance antisense oligonucleotide (ASO) performance, as outlined below.

First-Generation Oligonucleotides: Phosphorothioates and Methylphosphonates

Although methylphosphonates marked the initial attempt at modifying the phosphodiester backbone, phosphorothioates have become the cornerstone of early ASO development (Dias and Stein 2002). Methylphosphonate oligonucleotides feature a non-bridging oxygen replaced by a methyl group at each phosphate site, rendering them uncharged. This reduces solubility and limits cellular uptake due to reliance on endocytosis rather than passive diffusion (Blake et al. 1985; Tonkinson and Stein 1994). Despite their nuclease stability (Miller et al. 1979), methylphosphonates destabilize the native helix and fail to activate RNase H, which curbed their therapeutic utility.

Phosphorothioates, on the other hand, substitute one non-bridging oxygen with sulfur, enhancing resistance to nucleases and improving bioavailability (Dean and Bennett 2003; Behlke et al. 2005). This sulfur substitution introduces chirality, yielding Rp and Sp diastereomers with distinct properties: Sp is nuclease-resistant but destabilizes the helix and reduces binding affinity; Rp is more prone to enzymatic cleavage but better supports RNase H activity and duplex stability (Koziolkiewicz et al. 1995; Chan et al. 2006).

Despite their antisense efficacy, phosphorothioates often elicit off-target effects due to non-specific protein interactions, including with fibronectin, laminin, VEGF, and Mac-1 (Dias and Stein, 2002; Crooke, 2000). Some phosphorothioate ASOs that entered clinical use include ISIS 3521 (targeting PKC-α mRNA), G3139 (Oblimersen, targeting Bcl-2), and Fomivirsen (for CMV retinitis) (Geiger et al. 1998; Jansen et al. 2000; Mulamba et al. 1998).

Ongoing efforts to mitigate side effects led to the development of chimeric oligonucleotides, where phosphorothioate linkages are confined to the termini while retaining phosphodiester bonds in the central core, optimizing RNase H activity and minimizing toxicity (Eder and Walder, 1991). Additional strategies, such as incorporating C-5 propyne pyrimidines or C-7

modified purines, have improved target binding and stability (Buhr et al. 1996; McKay et al. 1996; Behlke et al. 2005).

Second-Generation Oligonucleotides: 2′ Modifications

To address the drawbacks of first-generation ASOs, second-generation oligonucleotides incorporate 2′ modifications that enhance nuclease resistance and target affinity. The most widely used include 2′-o-methoxyethyl (2′-MOE) and 2′-o-methyl (2′-OMe) modifications, both known for improved stability and stronger hybridization with target mRNA (Monia et al. 1993). While they do not induce RNase H activity (McKay et al. 1999), these ASOs inhibit translation via steric blockade (Johansson et al. 1994).

Early examples include ISIS 11158 and 11159, which block translation of ICAM-1 mRNA by targeting the 5′ cap region (Baker et al. 1997). A more recent candidate, OGX-011, is a 2′-MOE-modified gapmer targeting clusterin mRNA in prostate cancer (Chi 2005), while ISIS 104383 inhibits TNF-α expression. Gapmers, consisting of a central phosphorothioate "gap" flanked by 2′-modified regions, combine the benefits of RNase H activation and increased stability (McKay et al. 1999; Yu et al. 2004).

Further modifications—including C-5 propyne pyrimidine substitutions and 2′-O-allyl linkages—can significantly raise melting temperatures and enhance binding affinity, sometimes achieving IC50 values as low as 0.25 μM (Moulds et al. 1995). Generally, longer or bulkier 2′ substituents confer increased nuclease resistance, though often at the expense of target affinity, following the order: fluoro > methoxy > propoxy > pentoxy = deoxy (Monia et al. 1996). Hairpin loop attachments at the 3′ end also enhance stability (Kuwasaki et al. 1996).

Third-Generation Oligonucleotides

Third-generation ASOs introduce more extensive backbone alterations to optimize pharmacokinetics, nuclease resistance, and binding strength. Some retain the furanose ring (e.g., LNAs, PNs, FANAs), while others eliminate it entirely, as in morpholino oligomers (PMOs), peptide nucleic acids (PNAs), and tcDNA (Chan et al. 2006; Dias and Stein 2002).

PNAs are synthetic analogs with a neutral peptide-like backbone (Nielsen et al. 1991; Engholm 1993), enabling them to form highly stable duplexes and triplexes with complementary DNA or RNA without electrostatic repulsion (Koppelhus and Nielsen 2003). Their mechanism involves steric hindrance of translation or transcription rather than RNase H activity (Paulasova and Pellestor 2004).

PMOs replace the ribose with a morpholine ring and use phosphorodiamidate linkages, providing exceptional stability and low non-specific interactions. Like PNAs, they rely on steric interference to block translation (Summerton and Weller 1997; Chan et al. 2006). Techniques such as ARP conjugation and streptolysin O-mediated delivery have been employed to improve their cellular uptake (Nelson et al. 2005; Giles et al. 1999).

Phosphoramidate oligonucleotides (PNs) feature a 3′ amine substituting for the typical oxygen in the sugar-phosphate backbone, offering enhanced hybrid stability and target selectivity without requiring RNase H activation (Gryaznov et al. 1995; Chen et al. 1995). These can be further optimized with fluorinated modifications for even greater thermal stability (Schultz et al. 1996).

Locked nucleic acids (LNAs) feature a methylene bridge linking to the 2′ oxygen and 4′ carbon of the ribose ring, locking it into a favorable conformation. LNAs dramatically boost hybridization affinity and nuclease resistance, though they do not inherently support RNase H cleavage (Vester and Wengel 2004). When incorporated into gapmers with phosphorothioate cores, LNAs provide powerful RNase H activation and effective mRNA knockdown, particularly in the form of α-L-LNA, the most potent LNA stereoisomer (Kurreck et al. 2002; Fluiter et al. 2005).

ASO-Based Therapeutic Strategies for Liver Cancer

Das et al. (2009) demonstrated the anticancer efficacy of a c-raf-1-targeting antisense oligonucleotide (ASO) in hepatocarcinogenesis by suppressing the expression of c-raf-1 mRNA. In vitro studies revealed that this ASO reduced cell proliferation by 68%. Histopathological evaluation of liver tissues from treated animals revealed a marked decrease in preneoplastic lesions and hyperplastic nodules compared to carcinogen-exposed controls. Additionally, Mukherjee et al. (2009) reported biochemical changes in ASO-treated animals, including elevated cytochrome P-450 levels and reductions in glutathione S-transferase, UDP-glucuronosyltransferase activity, and malondialdehyde (MDA) levels.

In a separate investigation, Mukherjee et al. (2005) explored the impact of an IGF-II-specific ASO across various stages of hepatocarcinogenesis, from early preneoplastic changes to fully developed hepatocellular carcinoma (HCC). The study found that downregulation of IGF-II

expression not only delayed tumor progression but also suggested IGF-II as a potential early biomarker for liver cancer detection.

Cai et al. (1998) investigated the role of methionine adenosyltransferase (MAT) isoforms—encoded by MAT1A and MAT2A—in liver cell proliferation using gene-specific ASOs. Their work indicated that the type of MAT expressed in hepatic cells significantly influences growth characteristics.

Li et al. (2006) applied a 2′-O-methoxyethyl-modified phosphorothioate ASO to suppress STAT3 expression, a key transcription factor implicated in HCC. The ASO effectively reduced DNA-binding activity of STAT3 and lowered expression of angiogenic and anti-apoptotic markers such as VEGF, MMP-2, MMP-9, and survivin in HCC cell lines. In vivo, this led to enhanced apoptosis, reduced angiogenesis, and suppressed tumor growth, underscoring the therapeutic potential of targeting STAT3.

Similarly, Dai et al. (2005) used a survivin-specific ASO delivered via Lipofectamine™ 2000 in HepG2 cells. Their results highlighted survivin's pivotal role in HCC cell survival and proliferation, as its downregulation led to substantial tumor growth inhibition.

The pro-apoptotic effect of c-myc gene silencing was evaluated by Ebinuma et al. (2003), who used c-myc-targeting ASOs in multiple HCC-derived cell lines, including HCC-T, HepG2, and PLC/PRF/5. Western blot and morphological assessments confirmed that a significant decrease in c-myc mRNA expression triggered apoptosis, accompanied by a modest reduction in bcl-2 protein levels.

Wu et al. (2004) evaluated the anti-tumor efficacy of VEGF-targeting ASOs delivered intra-arterially in combination with lipiodol in a Walker-256 cell model. Their findings demonstrated that VEGF suppression curtailed tumor growth and angiogenesis.

ASO Applications in Other Cancers and Diseases

Numerous studies have explored ASO applications beyond liver cancer. Notably, ASOs have shown promise in treating:

- **Lung cancer** (Yasui et al. 2006; Hu et al. 2003; Datta et al. 2000; Olie et al. 2000; Tamm et al. 1998; Deveraux et al. 1997),
- **Colorectal cancer** (Saini et al. 2011; Qian et al. 2002; Ciardiello et al. 2000; Ichimura et al. 1996),
- **Pancreatic cancer** (Hotz et al. 2004; Nakano et al. 2001; Denham et al. 1998; Cheng et al. 1996; Aoki et al. 1995),

- **Ovarian cancer** (Popadeuk et al. 2006; Phillips et al. 2001; Sasaki et al. 2000; Alper et al. 1999; Ling et al. 1998; Cho-Chung et al. 1997; Metelev et al. 1994),
- **Prostate cancer** (Gritsko et al. 2006; Jhaveri et al. 2004; Fong et al. 2003; Iversen et al. 2003; Chi et al. 2001; Miyake et al. 2000; Balaji et al. 1997),
- **Breast cancer** (Tanabe et al. 2003; Paula et al. 2002; Chernicky et al. 2000),
- **Other malignancies** (Jansen et al. 2000; Webb et al. 1997).

ASOs have also demonstrated therapeutic efficacy in various non-oncologic diseases, including:

- **Diabetes mellitus** (Zemany et al. 2014; Toth 2013; Saonere 2011),
- **Cytomegalovirus (CMV) retinitis in AIDS** (de Smet et al. 1999),
- **Diabetic retinopathy** (Hnik et al. 2009; Alama et al. 1997; Rayburn and Zhang 2008),
- **Human papillomavirus (HPV)** (Alam et al. 2005; Galderisi et al. 1999),
- **Viral hemorrhagic fever (VHF)** (Bharti et al. 2009; Warfield et al. 2006; Saonere 2011),
- **Hepatitis B virus (HBV)** (Bai et al. 2013; Alama et al. 1997; Moriya et al. 1996),
- **Herpes simplex virus (HSV)** (Shogan et al. 2006; Alama et al. 1997),
- **Crohn's disease** (Monteleone et al. 2012),
- **Asthma** (Saonere 2011), and
- **Autism** (Bai et al. 2013).

Biodistribution of ASOs Based on Administration Route

Intravenous and Subcutaneous Administration

For systemic delivery, ASOs are primarily administered via intravenous (IV) or subcutaneous (SC) routes. Following administration, phosphorothioate-modified ASOs rapidly distribute from the bloodstream into tissues, predominantly through endocytosis, and remain in cells for extended durations, with half-lives ranging from 2 to 4 weeks (Yu et al. 2013; Crooke and Geary, 2013; Geary et al. 2009). SC injections achieve peak plasma levels within 4–5 hours, and animal models (e.g., monkeys) have shown near-complete bioavailability (Levin et al. 2007; Geary et al. 2009).

The distribution follows a biphasic pattern: a rapid distribution phase (minutes to hours) followed by a slower elimination phase lasting several weeks. In this phase, 2′-MOE ASOs showed a liver-to-plasma partition ratio of around 5000:1, consistent across multiple species (Geary et al. 2009).

Due to their chemical structure, phosphorothioate ASOs bind extensively to plasma proteins, particularly albumin (>80%), reducing renal clearance risks. This binding is weak (Kd ~150 μM), ensuring safety even at therapeutic doses (Yu et al. 2007; Levin et al. 2007). Conversely, uncharged ASOs like morpholinos and PNAs exhibit rapid renal clearance and limited tissue uptake (Iversen, 2007; Amantana and Iversen, 2005; McMahon et al. 2002).

Systemically administered ASOs, especially second-generation ones, are widely distributed to organs such as the liver, kidney, adipose tissue, bone marrow, and lymph nodes, showing consistent antisense activity (Yu et al. 2013; Crooke and Geary, 2013; Geary et al. 2009; Zhang et al. 2000). Studies suggest that slower infusion enhances hepatic uptake compared to bolus dosing, likely due to saturable uptake mechanisms (Geary et al. 2009). Interestingly, despite high hepatic ASO levels, functional antisense activity may not always correlate, suggesting intracellular barriers to action.

High muscle uptake has facilitated ASO development for diseases like Duchenne muscular dystrophy (DMD), utilizing exon skipping to correct Dystrophin gene mutations (Mendell et al. 2013; Deutekom et al. 2007; Aartsma-Rus et al. 2008). A similar exon-modifying approach has been applied to spinal muscular atrophy by targeting the SMN2 gene to restore SMN protein levels (Rigo et al. 2012; Passini et al. 2011).

Intrathecal Delivery

The blood-brain barrier (BBB) significantly limits ASO entry into the CNS, necessitating direct intrathecal (IT) delivery. Preclinical models have shown that IT administration enhances ASO penetration into brain and spinal tissues (Kordasiewicz et al. 2012; Rigo et al. 2012; Passini et al. 2011). Miller et al. (2013) reported that ASOs enter the CNS rapidly post-IT infusion, with a CSF half-life under one hour and limited systemic diffusion.

Compared to systemic delivery, IT administration results in higher central concentrations with minimal peripheral exposure (Rigo et al. 2014; Miller et al. 2013). Bolus IT injection also improves pharmacokinetics over continuous infusion (Miller et al. 2013). In both rodents and primates, IT-delivered ASOs efficiently reach neurons and exert strong antisense effects.

Delivery efficiency may further improve with nanocarrier systems. These carriers, including liposomes, micelles, dendrimers, and nanoparticles, enhance ASO transport across the BBB by facilitating endocytosis or controlled release (Caruso et al. 2012). Functionalization with brain-targeting ligands, neutral lipids (e.g., DOPC, DOPE), or PEGylated immunoliposomes offers site-specific delivery advantages (Xin et al. 2012; Martin-Banderas et al. 2011; Yang, 2010).

Cellular Uptake of Antisense Oligonucleotides

Due to their negatively charged nature, unmodified ASOs are unable to cross cell membranes unaided. Cellular uptake generally occurs through fluid-phase pinocytosis and adsorptive endocytosis (Chan et al. 2006; Dias and Stein, 2002), and is influenced by the ASO's structure, sequence, and temperature conditions (Dias and Stein, 2002). In both in vitro and in vivo settings, internalization can follow productive or nonproductive pathways—with only the former resulting in gene silencing (Geary et al. 2009).

ASO concentration plays a role in determining the uptake mechanism: low concentrations favor receptor-mediated endocytosis, while higher concentrations induce pinocytosis (Dias and Stein, 2002; Loke et al. 1989). Despite the loss of charge in some ASO types, such as peptide nucleic acids (PNAs) and phosphorodiamidate morpholino oligomers (PMOs), internalization remains inefficient due to poor interaction with cell surface proteins, leading to accumulation in endosomes or lysosomes (Grey et al. 1997; Chan et al. 2006).

Delivery vectors are often employed to enhance uptake and nuclear localization, offering protection from nuclease degradation and enabling lower therapeutic dosages (Lysik et al. 2003; Dias and Stein, 2002). First-generation phosphorothioate-modified ASOs improve binding to surface receptors and increase receptor-mediated uptake (Liang et al. 2014; Chan et al. 2006). Interestingly, certain cells like the MHT mouse hepatocyte line can internalize ASOs without transfection agents. Studies using this model helped identify the adaptor protein AP2M1, which facilitates clathrin-independent ASO uptake (Koller et al. 2011).

Liposomal formulations are among the most widely used vectors for ASO delivery. These vesicles are designed with a net positive charge to interact favorably with negatively charged cellular membranes (Dias and Stein 2002). Cationic lipids such as DOTMA and DOTAP are commonly used to enhance ASO encapsulation and internalization (Chan et al. 2006). Helper agents like DOPE and chloroquine promote endosomal escape by destabilizing the membrane under acidic conditions (Lysik et al. 2003; Ma et al. 1996; Felgner et al. 1996; Farhood et al. 1995; Bennett et al. 1992). At pH values below 5.5, DOPE encourages fusion with endosomal membranes, enabling ASO release into the cytosol (Fattal et al. 2004).

Other cationic polymers—such as poly-L-lysine, polyethyleneimine, dendrimers, and polyalkylcyanoacrylate nanoparticles—have also been investigated for ASO delivery (Dias and Stein, 2002). Cell-penetrating peptides (CPPs) represent another strategy, enabling energy-dependent ASO transport independent of traditional endocytic pathways (Chan et al. 2006; Dias and Stein, 2002). CPPs, typically fewer than 30 amino acids long, are conjugated to ASOs

via disulfide linkages. Examples include transportan, the HIV-TAT peptide, and penetratin (Nelson et al. 2005; Jarver and Langel 2004).

Ligand-ASO conjugation can also increase tissue specificity. For instance, conjugating ASOs with triantennary GalNAc ligands has been shown to increase hepatocyte uptake by roughly tenfold, while reducing off-target distribution (Prakash et al. 2014).

Recent Advances in ASO-Based Therapies

Oligonucleotide-based therapeutics have ushered in a promising era for targeted treatment strategies. Despite robust preclinical data, clinical translation has been relatively slow due to multiple delivery and efficacy challenges. So far, only a few ASOs have reached the clinical stage. One such example is a liposomal c-raf ASO formulation currently undergoing clinical evaluation (Zhang et al. 2009). Mipomersen, targeting ApoB100, remains the only FDA-approved ASO, used since 2013 to treat homozygous familial hypercholesterolemia.

Interest in ASO therapies has been renewed, with recent clinical trials highlighting their potential. In patients with advanced malignant melanoma characterized by high bcl-2 expression, combination therapy with dacarbazine and a bcl-2-targeted ASO significantly enhanced tumor cell apoptosis and suppressed bcl-2 protein levels (Jansen et al. 2009).

ASOs targeting microRNAs—specifically miR-21 and miR-221—have shown promise in pancreatic cancer models. When combined with gemcitabine, these ASOs heightened cell death and reduced proliferation in HS766T cell lines. Apoptosis rates increased by up to sixfold, with concurrent elevation of tumor suppressor proteins (Jong-Kook et al. 2009).

In another notable study, phosphorothioate-modified ASOs against insulin-like growth factor II (IGF-II) mRNA significantly inhibited tumor development in a rat hepatocarcinoma model. In 40% of the treated animals, no hepatic tumors were detected, while the remainder showed significantly reduced lesion sizes—by 64% and 53% for exon-1 and exon-3 targets, respectively (Ghosh et al. 2014). Additionally, serum LDH activity and tumor burden were dose-dependently decreased. Cantide, an ASO targeting human telomerase reverse transcriptase (hTERT), significantly extended survival in tumor-bearing mice (Yang et al. 2012).

Targeted ASOs also proved effective in fibrotic models. In an ex vivo study, ASOs targeting ARK-5 disrupted collagen structure and suppressed its expression by inducing exon skipping in the precursor mRNA (Karkampouna et al. 2014). Moreover, ASOs directed at the TNF

receptor reduced TNF-induced toxicity and protein levels in major organs (Hauwerneiren et al. 2014).

Despite over three decades of research, the clinical progress of ASOs remains limited, underscoring the numerous obstacles that hinder their full therapeutic potential. Overcoming these challenges—particularly improving tissue-specific delivery, enhancing pharmacokinetic profiles, and optimizing molecular design—will be crucial for translating ASO-based therapies into more widespread clinical use.

Chapter 3

Research envisaged

(Aim and Objectives)

Research envisaged

Globally, hepatocellular carcinoma (HCC) carries on as a key driver of cancer-related mortality. Chemotherapeutic intervention is the only therapy option for HCC, a high-mortality illness that typically shows no symptoms in its early stages and is most often detected at a late stage. Its chemotherapeutic alternatives, however, fall well short of expectations. Most anticancer medications have a high cytotoxicity level. In contrast to previous chemotherapeutics, genetic treatments, such as antisense oligomer (ASO), shown a preponderance of HCC inhibitory potential with negligible toxicity.

In order to inhibit HCC in rats, we have examined the effectiveness of commercial docetaxel formulations (DXT) and H-Ras targeted genetic treatment. Additionally, utilizing intravenous administration of the corresponding ASO in chemically caused HCC rats, an attempt was made to block mutant *H-Ras* or *c-raf.1* gene expressions frequently linked to HCC development and progression in order to comprehend the effectiveness and mechanistic intervention of the treatments.

AIM

Antisense oligonucleotides/oligomers against mutated *H-Ras* gene may inhibit HCC and delay the progress of chemically induced HCC development in rats.

OBJECTIVES

The effectiveness of taxotere®, a commercially available formulation of docetaxel, and *H-Ras*-targeted gene therapy in preventing carcinogen-induced HCC in rats was compared in this study. To make the conclusion, a number of biochemical investigations, histological analysis, pharmacokinetic estimation, histochemical assessment, and morphological characteristics were conducted in conjunction with confocal microscopic analysis and electron microscopic observation.

Furthermore, the formation and progression of HCC have long been linked to mutations in the

Ras-Raf-MAP (mitogen-activated protein) kinase pathway, namely the H-Ras mutation (located in codon 61 at position 12) and the Raf-1 (*c-raf.1*) mutation (at CRAFP261A) (Sun et al. 2022; Gedaly et al. 2010; Das et al. 2010).

For this investigation, two antisense oligomer sequences have been chosen: one against H-Ras (also written as H-ras) (ASO1) and one against c-raf.1 (ASO2). We selected ASO2 from the reported literature. Using human tumor xenograft animal models, a report showed that ASO selectively inhibited the expression of the c-raf-1 kinase gene and the growth of tumors in cell culture and in vivo (Monia et al. 1999). To compare and comprehend their therapeutic potential and mechanistic approaches to inhibit HCC in rats, we attempted to use selective phosphorothioate backbone-modified antisense oligomers (please refer to the Materails and Methods section for the oligomer sequences) to inhibit mutated *H-Ras/c-raf.1*-overexpression individually in rats.

Pointed objectives

- To design and develop both labeled and unlabeled phosphorothioate H-Ras sense and antisense oligonucleotides.
- To see how hepatic neoplastic cells and normal cells behave once the mutated *H-Ras* gene is blocked by the experimental antisense oligomer.
- To compare the hepatic architectures of the carcinogen-treated rats, the carcinogen-treated rats treated with H-Ras antisense oligomer, the carcinogen-treated rats treated with a commercial formulation (Taxotere), and the normal untreated rats.
- To compare *H-Ras* gene and various hepatic marker protein expressions in the experimental rat livers by in situ hybridization and immuno-histochemical methods, respectively.
- To compare the hepatic ultrastructure of the experimental rats.
- To assess the hepatic marker enzyme and caspase activities in the experimental rats.
- To compare HCC inhibition by blocking mutated *H-Ras* and *c-raf.1* genes using the respective antisense oligomers.

Chapter 4

Plan of work

Plan of work

- A thorough literature review for better understanding of the research proposal, various required methodologies, and exploring the insight about the research investigation.
- Both 6-carboxyfluorescein (FAM) labeled and unlabeled phosphorothioate H-Ras sense and antisense (PS-ASO) oligonucleotide designing, development and characterization.
- In vitro hemolysis study of PS-ASOs
- Hepatic PS-ASO uptake in normal rats
- Pharmacokinetic profiles and biodistribution study of PS-ASO
- Effect of PS-ASO treatments on some perfused organs of normal rats
- Antineoplastic in vivo study
- Hepatic macroscopic examination
- Hepatic microscopic examination
- H-Ras mRNA expression in experimental rats treated with PS-ASO and Docetaxel by in situ hybridization
- Immunohistochemical examination of p53, Hep Par 1, CK7, and CD15 in experimental rats treated with PS-ASO and Docetaxel by in situ hybridization
- Scanning electron microscopic evaluation of livers of the experimental rats
- Effect of PS-ASO /DTX on ALK, SGPT, and SGOT activities in the experimental rat livers
- Effect of PS-ASO/DXT on caspase 3 and caspase 9 activities in experimental rat livers
- Studying the effect of HCC inhibition by blocking mutated *H-Ras* and *c-raf.1* genes using the respective antisense oligomers, after their characterizations by atomic force microscopy (AFM)
- Antisense oligomer (against mutated *H-Ras* and *c-raf.1* genes) characterization by atomic force microscopy (AFM)
- In vitro cell cytotoxicity assay in HepG2, Huh7, Wrl68 and Chang liver cells

- Cellular apoptosis assay
- Mitochondrial membrane potential (MMP) study by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1)
- In vivo hepatic accumulation of 6-carboxyfluorescein (FAM)-labeled ASOs
- Effect of the treatments on normal and carcinogen-induced HCC rats
- Microscopic evaluation
- In-situ hybridization study of mutated *H-Ras/ c-raf.1* gene expression
- Immunohistochemical evaluation p53, Hep Par 1 and HSP70 proteins after the treatments of ASO against mutated *H-Ras/ c-raf.1* in the experimental rats
- Hepatic caspase 3/9 activities after the treatments of ASO against mutated *H-Ras/ c-raf.1* in the experimental rats
- Hepatic cytosolic alanine aminotransferase (ALT) and microsomal aspartate aminotransferase (AST) enzyme assays after the treatments of ASO against mutated *H-Ras/ c-raf.1* in the experimental rats
- Data compilation and statistical analysis
- Research publication
- Presentation of the work in seminars/conferences
- Thesis preparation and submission

Diagrammatic representation of the entire work has been described in the following two figures (Fig. 4.1 and Fig. 4.2). Fig. 4.1 describes how carcinogen-treated rats, the carcinogen-treated rats treated with H-Ras antisense oligomer, the carcinogen-treated rats treated with a commercial formulation (Taxotere) modulated chemically induced HCC in rats by modulating various morphological and biochemical parameters. Further, a comparative HCC inhibition by blocking mutated *H-Ras* and *c-raf.1* genes using the respective antisense oligomers showed that ASO against the mutated Ras was potentially more effective to inhibit HCC in rats (Fig. 4.2).

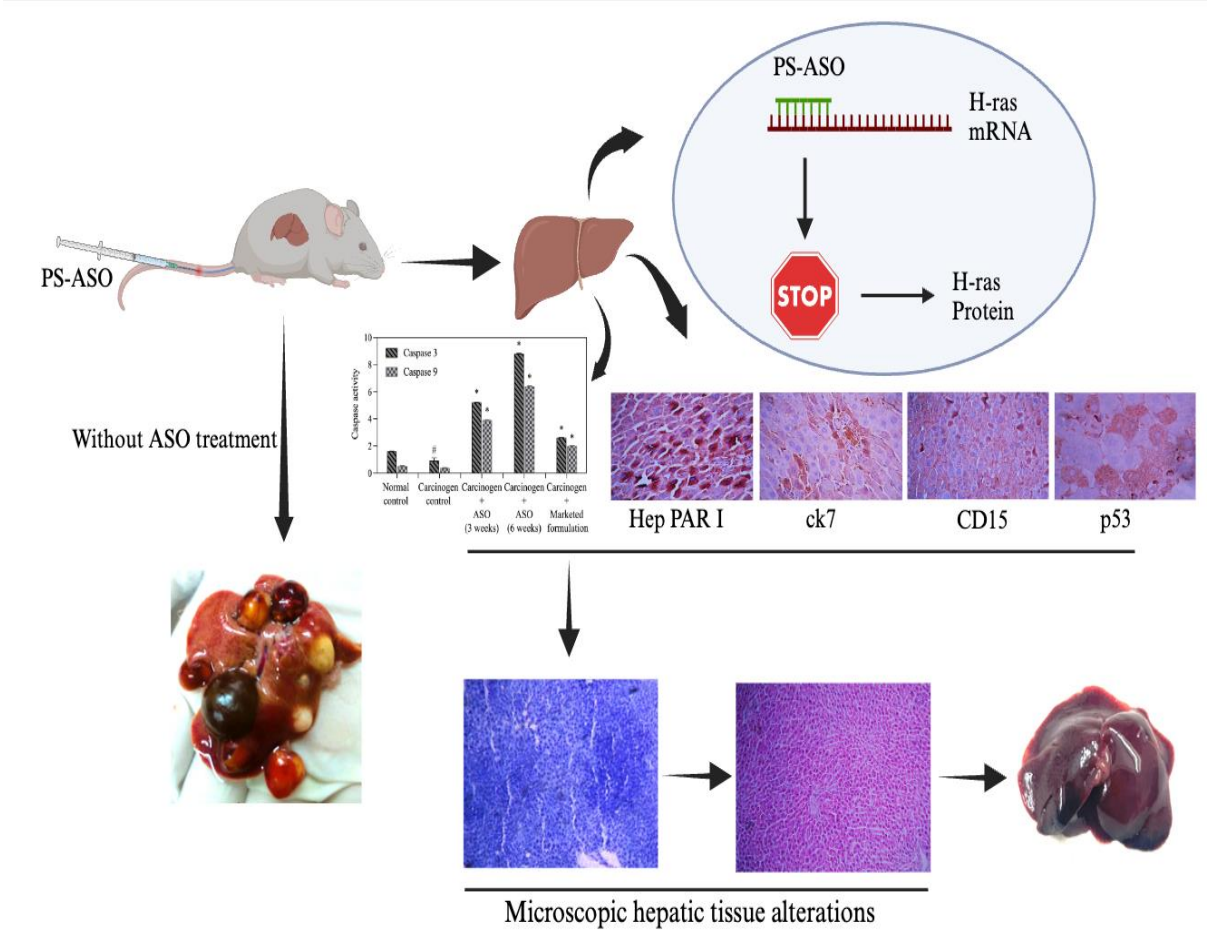

Fig. 4.1. Effect of PS-ASO treatment in carcinogen-treated rats treated with antisense oligomer against mutated *H-Ras*, and the carcinogen-treated rats treated with a commercial formulation (Taxotere) based on various biochemical and morphological investigations. PS-ASO treatment showed remarkable HCC inhibitory effect than Taxotere in rats

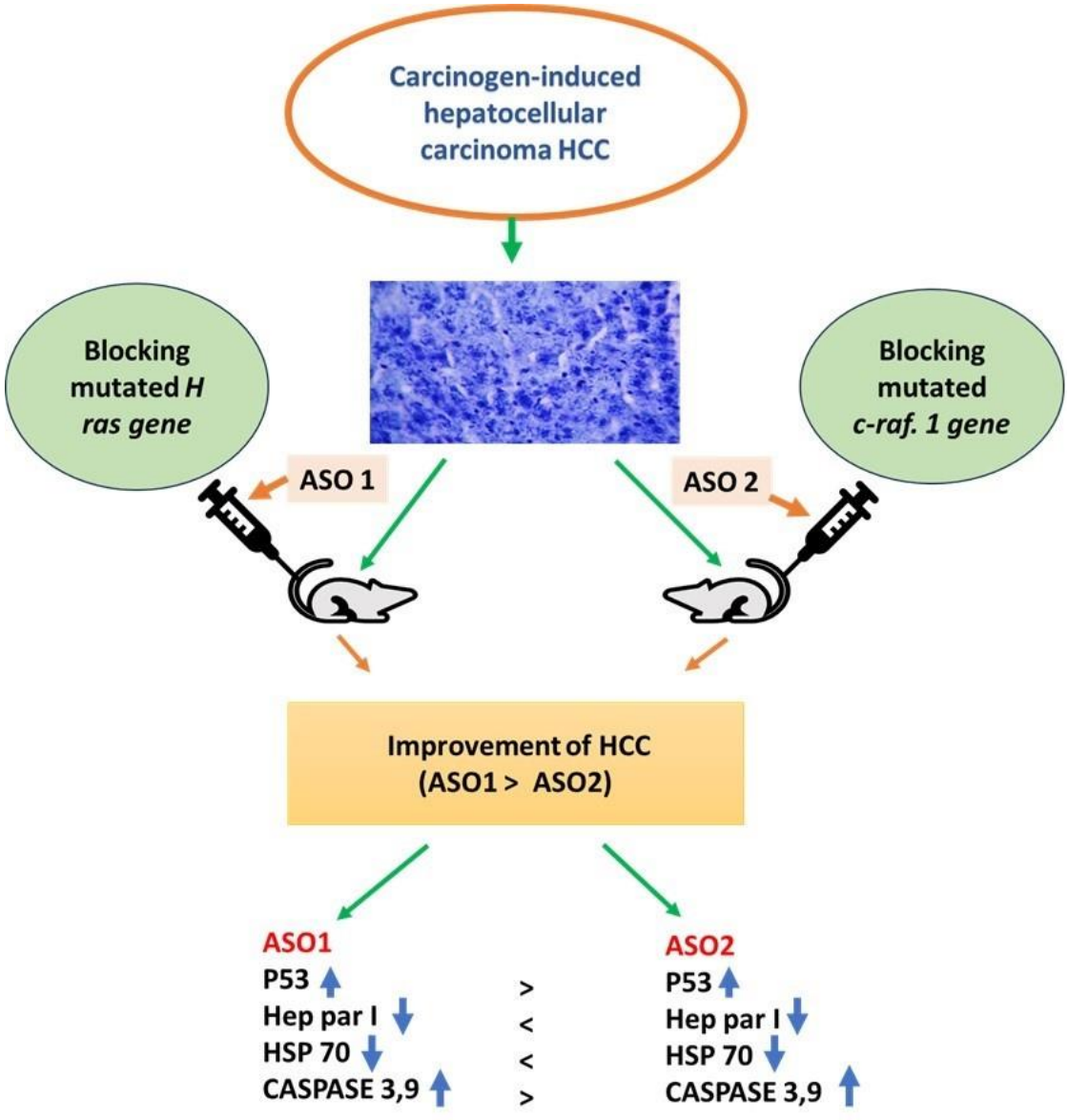

Fig. 4.2. A comparative HCC inhibition by individually blocking mutated *H-Ras (H ras)/ c-raf.1* genes using the respective antisense oligomer showed that ASO against the mutated *H-Ras* effectively inhibited HCC in rats, by altering several biochemical and histological parameters.

Chapter 5

Materials, equipment and methods

Material and methods

Chemicals:

List of chemicals used and manufacturers

Chemical/Item	Manufacturer
5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (MitoProbe JC-1 assay kit)	Thermo Fisher Scientific, Waltham, MA, USA
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich, Bengaluru
Agarose powder	Hymenia
Ethidium bromide	Sigma-Aldrich, Bengaluru
Caspase-3/9 assay kit	BioVision colorimetric caspase assay kit, BioVision, Milpitas, California, USA.
Annexin-V FITC (fluorescein isothiocyanate)/ propidium iodide (PI) staining kit (FITC annexin V/dead cell apoptosis kit	Thermo Fisher Scientific, Waltham, MA, USA
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma-Aldrich, Bengaluru
Taxotere®	Emcure Sanofi, Pune, India.
Diethyl Nitrosamine (DENA)	Sigma-Aldrich, Bengaluru
2-Acetylaminofluorene (2-AAF)	Sigma-Aldrich, Bengaluru.
Aspartate aminotransferase (AST) (Aspartate Aminotransferase (AST/GOT) Activity Assay Kit)	Coral Clinical Systems, Goa, India.
Alanine aminotransferase (ALT) (Aspartate Aminotransferase (AST/GOT) Activity Assay Kit)	Coral Clinical Systems, Goa, India.

Glutamate oxaloacetate transaminase (SGOT) (Aspartate Aminotransferase (AST/GOT) Activity Assay Kit)	Coral Clinical Systems, Goa, India.
Glutamate pyruvate transaminase (SGPT) (Aspartate Aminotransferase (AST/GOT) Activity Assay Kit)	Coral Clinical Systems, Goa, India.
Alkaline phosphatase (ALK) assay kit	Coral Clinical Systems, Goa, India
Modified Lowry protein assay kit	Thermo Scientific, Rockford, IL 61105 USA
Tris base	Sigma-Aldrich, Bengaluru
Na-dithionite	Sigma Aldrich, Bengaluru, India.
DIG Nucleic Acid Detection Kit	Roche Diagnostics Asia Pvt Ltd, Singapore, 20
Proteinase K	Sigma-Aldrich, Bengaluru
EDTA	Process Chemical Industries, Kolkata, India
Sodium Dodecyl Sulfate	s.d.fine-chem Limited, Mumbai, India
Haematoxylin	GlaxoSmithKline Pharmaceutical Limited, Mumbai, India
Eosin	Qualigens fine chemicals, Mumbai, India
Periodic acid	Sigma-Aldrich, Bengaluru
Schiff base	Sigma-Aldrich, Bengaluru
Toluidine blue	Sigma-Aldrich, Bengaluru
ROCHE DIG-High Prime DNA Labeling and Detection kit	Roche, Penzberg, Germany

Primary antibodies (anti-Hep Par 1, anti- CD15, anti- CK7, and anti-p53)	Thermo Fisher Scientific, Kolkata, India
Anti-HSP70 primary antibody	Bio Bharati LifeScience Private Limited, Kolkata, India
Horseradishperoxidase-conjugated anti-mouse/ anti-rabbit secondary antibodies	Dianova, Hamburg, Germany
Every other chemical utilized was of analytical or molecular biology grade.	

Important equipment used for this investigation

Equipment	Manufacturer
Scanning electron microscope	
Transmission electron microscope	JEOL JEM 2100 HR, Tokyo, Japan
Atomic force microscope	
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry	Shimadzu, Kyoto, Japan
Gel electrophoresis system	H.L Scientific Industry Pvt. Ltd
Dynamic Light Scattering system and zetasizer	Malvern, UK
Transilluminator fitted with a camera.	Imagene Innolab LLP

Oligonucleotide Sequences Utilized

A 21-mer phosphorothioate-modified antisense oligonucleotide with the sequence 5′-UCCCACACCGACGGCCCCCA*C-3′ was employed in this study, both with and without a 5′-end label using 6-carboxyfluorescein (6-FAM) (Biotech Desk, Hyderabad, India). *For gene expression assays, a digoxigenin (DIG)-labeled antisense RNA sequence,* 5′-TCCCACCCGACGCCCCCC-3′ , and its complementary sense strand were synthesized by Eurofins Genomics (Bangalore, India), incorporating dUTP for labeling.

To compare target specificity against mutant *H-Ras* (carrying a codon 61 mutation at position 12) and *c-raf-1* genes, two phosphorothioate-backbone-modified oligonucleotides were selected: a 21-mer ASO (5′-T*CCCA*C*A*C*CG*A*CG*G*CG*C*CC*A*C*-3′, designated ASO1) and a 20-mer ASO targeting *c-raf-1* (5′-T*CCCG*C*CT*G*T*G*A*C*A*T*GCA*T*T*-3′, designated ASO2). These sequences, also synthesized by Eurofins Genomics, are designed to selectively downregulate mutated *H-Ras* and *c-raf-1* gene expression.

Additionally, DIG-labeled antisense RNA probes were prepared for both target transcripts: 5′-U*U*A*CG*U*A*C*A*G*U*G*U*CCG*C*C*C*U*-3′ for c-raf-1 mRNA and 5′-U*CCCA*C*A*C*CG*A*CG*G*CG*C*CC*A*C-3′ for the mutant H-Ras transcript, along with their respective sense strands, also obtained from Eurofins Genomics.

The commercial chemotherapeutic agent docetaxel (Taxotere®, Emcure Sanofi, Pune, India) was sourced through Aldine, Kolkata, India, for combination studies.

Methods

Physicochemical Characterization of Antisense Oligomers

Mass Spectrometry

The molecular mass of the phosphorothioate-modified oligomers was done using electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) techniques. These procedures were conducted following previously reported methodologies (Nikcevic et al. 2011; Apffel et al. 1997), and involved analysis of mass-to-charge (m/z) ratios.

Zeta Potential Measurement

To assess the surface charge of the ASOs, dynamic light scattering (DLS) was used. Two microliters of each oligonucleotide preparation were mixed with molecular-grade water (Millipore), briefly centrifuged, and transferred into a quartz cuvette (1 mL volume) for zeta potential measurement as per the method of Shaw et al. (2017).

Atomic Force Microscopy (AFM)

Surface topography and morphology of ASOs targeting mutant H-Ras and c-raf-1 were analyzed using AFM. A 50 µL aliquot of each ASO sample (10 µg/mL) was dropped on freshly cleaved mica, incubated for 10 minutes, washed gently, dried under nitrogen, and imaged in air using silicon cantilevers (Herrmann, 2021). The acquired images were processed with PicoView software.

Agarose Gel Electrophoresis

Electrophoretic analysis was conducted using 2% agarose gel prepared in 1×TAE buffer (Tris-acetate-EDTA). We then added ethidium bromide solution (10 µL) to the gel prior to solidification. Oligomer samples were combined with a loading buffer, denatured at 95°C for 1 minute, cooled, and loaded into wells alongside a DNA ladder. Electrophoresis was performed at 100 V for 45 minutes. Post-run visualization was done using a UV transilluminator with an integrated camera system.

Transmission Electron Microscopy (TEM)

TEM was employed to evaluate the internal ultrastructure of the ASOs. Samples were suspended in Milli-Q water and deposited onto 300-mesh carbon-coated copper grids. After air-drying, imaging was carried out using a JEOL JEM 2100 HR transmission electron microscope (Tokyo, Japan).

Selected Area Electron Diffraction (SAED)

SAED analysis was performed to assess the crystallinity of the ASOs. The diffraction patterns were captured using the JEOL JEM 2100 HR TEM system, following the approach described by Zou et al. (2004).

In Vitro Hemolysis Assay

Investigation of ASO-governed hemolysis in vitro

Using the methodology of Kumari et al. 2023 (Leena Kumari et al. 2023), in vitro hemolysis was investigated. A heparinized tube containing the blood drawn from the tail vein was centrifuged at 2000xg for 5 minutes at 4 °C. Following the removal of the supernatant, the

erythrocytes were rinsed three times with phosphate buffer-saline (PBS) (pH 7.4). After adding 190 µL of erythrocyte suspension (2%) in PBS to each well of a 96-well plate, 10 µL of ASO suspension at varying doses (5–100 nM) was added. They were thereafter incubated for one hour at 37 °C with a gentle shaking. Centrifugation was used to gather the erythrocytes that had not undergone hemolysis (10000x g for 5 min). At 570 nm, the supernatant's absorbance was calculated. A 100% hemolytic blood sample's absorbance was measured. The following formula was used to determine the proportion of hemolysis:

$$Hemolysis\ (\%) = \frac{(\text{absorbance of the experimental sample} - \text{absorbance of a solution with 0\% hemolysis})}{(\text{absorbance of a solution with 100\% hemolysis} - \text{absorbance of a solution with 0\% hemolysis})}\ X\ 100$$

In Vitro Cytotoxicity Assay

To assess the cytotoxic effects of the antisense oligonucleotides (ASOs), 1.5 × 10^4 cells per well (HepG2, Huh7, Chang liver, or WRL-68) were seeded into 96-well plates containing DMEM with 10% FBS (fetal bovine serum) as supplement, along with 50 IU/mL penicillin G and 50 µg/mL streptomycin and incubated in CO_2 incubator at 37°C with 5% CO_2. Following 48-hour incubation with ASOs, new media without FBS was added after proper washing the cells with ice-cold phosphate buffer (pH 7.4). Then 20 µL of MTT solution (5 mg/mL in PBS) was poured to each well and incubation was continued for the next 4 hours. The resulting formazan crystals were dissolved using 100 µL of DMSO, and absorbance was measured at 540 nm using an ELISA microplate reader (Bio-Rad, CA, USA), following the method described by Chakraborty et al. (2020).

IC_{50} values were calculated using OriginPro software based on data from three independent experiments.

Evaluation of mitochondrial membrane potential (MMP) using JC-1 staining by Flow cytometry

Using the mitochondria-specific dye JC-1, the changes in MMP, one of the hallmarks of apoptosis, were examined. Then, using a flow cytometer and the JC-1 kit (MitoProbe JC-1 assay kit, Thermo Fisher Scientific, Waltham, MA, USA), the changes in red (J aggregates

form) to green fluorescence (for monomers) were quantitatively determined (Chakraborty et al. 2020, 2023). ASO1/2 was applied to HepG2 and Huh7 cells for 48 hours.

In vivo experiments

All of the in vivo tests were conducted on male Sprague-Dawley (SD) rats (For the first study, 100-120 g; and for the second investigation, 130-145 g body weight). The animal tests were conducted after being approved by the University's animal ethics committee (approval no.1704/03/2020). Six rats per cage (if not otherwise mentioned) were kept in an environment with a regular photo period every day and a consistent temperature of 22±0.5°C and humidity of 55%.

Dose determination study

Both labelled and unlabelled PS-ASOs (0.5 mg/kg body weight–5 mg/kg body weight) were used in the dose escalation study. ASOs were suspended in water for injection (I.P.) and sterilized through sterilized membrane syringe filter (HiMedia, Kolkata, India). This process caused a loss of approximately 20% ASOs as observed by us. Hence, we added 20% overages of ASOs to the filtration sample. The results showed that experimental animals exhibited toxicity (without death) at a maximum dose of 4 mg/kg, as evidenced by physical symptoms like sedation and levels of biochemical parameters like urea, creatinine, SGPT, ALK, and SGOT. Thus, we have chosen a safer unlabeled PS-ASO dosage of 2 mg/kg body weight, or the equivalent of labelled PS-ASO. Next, we injected the FAM-labeled PS-antisense oligomers into the rats' tail veins and conducted pharmacokinetic and biodistribution studies. Blood levels of PS-ASOs are known to be extremely stable (Cunningham et al. 2001).

In vivo cellular internalization of FAM-labeled ASO

Three male SD rats from each group were given FAM-labeled ASO (2 mg/kg bodyweight) via the tail vein. Under anesthesia, the animals were sacrificed. At 2, 6, and 8 hours, liver samples were taken, and no extra dyes were used throughout the slide preparation process. We used a confocal microscope to view the slides. Photographs were taken.

Development of calibrations curves for FAM-labeled PS-ASO estimation from blood, liver, kindney, lung and spleen of the experimental animals.

For the estimation of FAM-labeled PS-ASO from blood and the other tissues (liver, kidney, lung and spleen), blood was taken and tissue samples were removed from normal rats. The

blood taken in heparinized tube and plasma was obtained by centrifuging the blood at 2000 x g in a refrigerated centrifuge. The tissue homogenate was prepared by homogenizing pre-weighed tissue samples in 1.15% cold KCl. The known concentrations (1, 2, 3, 4, and 5 µg/ µl) of the labeled PS-ASO were added to blood plasma and the respective tissue

Fig. 5.1. ASO injection in rat tail-vein

homogenates of rats. They were read spectrofluorimetrically by excitation at 490 nm and emission at 515 nm, and the data were plotted for developing the respective calibration curves.

In vivo plasma pharmacokinetic (PK) and tissue distribution study

Three groups of normal rats, each comprising three SD male rats, were separated. Rats in one group got an injection of FAM-labeled PS-ASO (2 mg/kg bodyweight) via the tail vein (Fig. 5.1), while rats in the other group received an injection of Taxotere® (2 mg/kg bodyweight) via the tail vein (Shaw et al. 2017). No therapy was given to the third group, which consisted of normal animals. Blood samples were obtained at 2, 4, 6, and 8 hours. During the experimental time periods, a terminal cardiac puncture was performed under deep anesthesia to obtain around 1.0 mL of blood in a microcentrifuge tube. After separating the plasma without the use of an anticoagulant, it was centrifuged for six minutes at 3000 rpm. Normal serum was used as a control when the serum samples were spectrofluorimetrically read (Ghosh et al. 2014), using normal respective tissue homogenate as control.

The liver, kidney, lungs, and spleen were then removed from the three animals in each group after they were killed at two, six, and eight hours. Using the normal tissue homogenate as a control, a weighted part of each tissue sample was homogenized in cold 1.15% KCl, diluted, and the ASO level was measured spectrofluorimetrically Ghosh et al. 2014). HPLC was used to quantify DTX, as previously reported (Kazemi et al. 2020). After that, many PK parameters were identified. Additionally, all 8-hour FAM-treated tissue samples were processed, DAPI-counterstained, and examined using confocal microscopy (Ghosh et al. 2014).

Determination of treatment effect on normal and carcinogen-induced HCC rats

Sprague-Dawley male rats were given a continuous 16-week treatment of 0.05% (w/w) 2-acytylamminofluorene (2AAF) as a complete carcinogen in their food, which resulted in hepatocarcinogenesis (Mukherjee et al. 1996). In addition to water *ad libitum*, they were given a carcinogen-mixed food at 10 a.m. and the basal diet (Mukherjee et al. 1996) at 3 p.m. The following groups, each with six animals, were created from the animals for the determination of treatment effect on normal and carcinogen-induced HCC rats. All animals had free access to water.

Group A: Carcinogen control rats were fed 0.05% (w/w) 2AAF for 16 weeks in a row as a complete carcinogen.

Group B: Untreated, normal animals were given simply a basal feed and free-access to water.

Group C: For 16 weeks, the rats were given the same dosage of 2AAF in their food. They were also given ASO (without FAM labeling) three times a week for three weeks (from week 31 to week 33) at a dose of 2 mg/kg bodyweight, which was given through the tail vein.

Group D: The rats were given the same antisense ASO therapy (without FAM labeling), as well as non-carcinogenic basal diet and free-access to water.

Group E: The rats were given the same dose of 2AAF in their diet for 16 weeks, along with the treatment of PS-ASO (without FAM labeling) three times a week for 6 weeks (from week 31 to week 36), at a dose of 2 mg/kg bodyweight, administered through the tail vein.

Group F: The rats were given the same treatment of antisense PS-ASO (without FAM labeling), along with the basal diet (without carcinogen) and water at will.

Group G: The rats were given the same dose of 2AAF in their diet for 16 weeks, along with the treatment of commercial formulation (Taxotere®) three times a week for 6 weeks (from week 31 to week 36) at a dose of 2 mg/kg bodyweight (Shaw et al. 2017).

Group H: The rats were given the same commercial formulation therapy, along with basal diet devoid of carcinogen and free access to water.

Fig. 5.2. Organ separation

In the case of the comparison of the corresponding ASO efficacies against the mutated *H-Ras* gene and *c-raf.1* gene, respectively (the second investigation), the animals (SD) were split up into five groups, which were as follows. The treatments the animals got are listed below.

Group A: The animals in this group were given simply a basal diet (Mukherjee et al. 1996) and free access to water.

Group B: Carcinogen control rats were fed 0.05% (w/w) 2AAF as a complete carcinogen for 16 weeks in a row (Mukherjee et al. 1996).

Group C: In addition to receiving the same dosage of 2AAF in their diet for 16 weeks, rats were treated with unlabelled ASO against the mutant H-Ras mRNA three times a week for six weeks (from the 31st to the 36th week) at a dose of 2 mg/kg body weight, which was given through the tail vein.

Group D: For 16 weeks, the rats were given the same dosage of 2AAF in their diet. They also received ASO therapy against c-raf.1 mRNA (without FAM tagging), which was given three times a week for six weeks (from the 31st to the 36th week) at a dose of 2 mg/kg body weight through the tail vein.

Since our just earlier depicted study and previous investigation demonstrated that ASO

treatments with the same dose range had no harmful effect in normal rats, no ASO control trial was carried out in normal animals in this case.

At the 38th week following the experiment's commencement, all animals were anesthetized before being dissected. Before being killed, the animals were left without food for 12 hours. All of the liver samples and other tissues were removed (Fig. 5.2), macroscopically inspected, and stored properly for additional examination. There was no discernible tumor growth in any other organ than liver and lungs.

Every tissue sample was sliced into small pieces. Normal rat liver samples that have undergone different treatments were assessed for SEM, enzymatic, and histological examinations. The remaining samples underwent enzymatic, immunohistochemical, SEM, histological, and in-situ gene expression studies. The sole analysis performed on tumored lungs was histopathological. Until they were needed again, tissue samples were kept at -80°C after being snap-frozen in liquid nitrogen at -196°C. Before being used, the samples were thawed.

Macroscopic observation and evaluation of the experimental tissues

Macroscopic analysis of experimental rat tissues was performed to determine whether tumor growth or hyperplastic nodular growth was present.

Microscopic examination

Following tissue sample fixation in 10% neutral buffered formalin, paraffin-embedded tissues were sectioned using a microtome (6 µm thick). The tissue sections were examined under an optical microscope after being stained with toluidine blue (TB), hematoxylin-eosin (HE), and periodic acid-Schiff (PAS) following the removal of the wax. Numerous hepatic changed lesions, both preneoplastic and neoplastic, were recognized as hepatic altered foci (HAF). Using a Zeiss light microscope, the numbers and diameters of the lesions were measured, and Axio Vision software 4.7.1 was used for analysis.

In-situ gene expression

By using the digoxigenin (DIG)-labelled antisense and sense oligomers of the gene on hepatic sections, the expression of the *H-Ras/ c-raf.1* mutated gene was examined in both carcinogen-

treated rats and rats treated with carcinogen and given ASO/DXT (where applicable) treatments. This was done essentially using the methodology of Mukherjee et al. (2005). The slides of liver sections were treated with the denatured probes at 37°C for overnight for hybridization, and then washed. The slides were further incubated with anti-DIG-polyclonal antibody conjugated with alkaline phosphatase for two hours following stringency washing with saline sodium citrate and blocking with a 0.5% blocking solution at room temperature. Nitroblue tetrazolium/ bromochloroindolylphosphate (NBT/BCIP) staining was done. The slides were then observed under microscope.

Immunohistochemical evaluation

The immunohistochemical investigations done here facilitated the determination of specific protein expressions in the hepatic tissues. Using the primary antibodies (anti-Hep Par 1, anti-CD15, anti-CK7, anti-p53, anti-Hep Par 1 antibodies from Thermo-Scientific) and anti-HSP70 antibodies (Bio Bharati LifeScience Private Limited, Kolkata, India), immunohistochemical analyses were conducted in compliance with previously published protocols (Hanif and Mansoor, 2014, Rullier et al. 2000, Karabork et al 2010, Torii et al. 1993, Li et al. 2021). The slides were washed and treated with horseradish peroxidase-conjugated anti-mouse/anti-rabbit secondary antibodies (Dianova, Hamburg, Germany) following the monoclonal antibody incubation. The studies made it easier to identify particular proteins in the liver tissues.

SEM analysis

Samples of liver tissue were taken from the treatment, carcinogen control, and normal groups of rats. A solution of 2.5% glutaraldehyde in PBS (pH 7.2) was used to fix the samples for an hour at 4°C. A previously described technique (Ghosh et al. 2014) was then used to prepare the tissue samples, and they were examined using a scanning electron microscope (JSM Electron microscope, JEOL, Tokyo, Japan) at an operating voltage of 15 kV. Every sample was photographed while scanned at different magnifications.

Serum level of glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT), and alkaline phosphatase (ALK)

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From rat tail vein, blood samples were drawn. No anticoagulant was added to it. The samples were left to stand for 30 minutes. After centrifuging the samples for 10 minutes at 2500 rpm, the serum was extracted and used, in accordance with the manufacturer's instructions, to analyze serum glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT), and alkaline phosphatase (ALK) using a commercial kit (Coral Clinical Systems, Goa, India).

Hepatic cytosolic alanine aminotransferase (ALT) and microsomal aspartate aminotransferase (AST) enzyme assays

Following the described procedure, the cytosolic and microsomal fractions of the experimental hepatic tissue samples were separated after they had been homogenized in a cold 1.15% KCl solution. The Aspartate Aminotransferase (AST/GOT) Activity Assay Kit (Coral Clinical Systems, Goa, India) was used to measure ALT activity from the cytosolic fraction and AST activity from the microsomal fraction in accordance with the manufacturer's instructions.

Caspase 3/9 activities

A cold 1.15% KCl solution was used to homogenize the hepatic tissue samples. Following the manufacturer's instructions, a colorimetric caspase assay kit (BioVision, Milpitas, California, USA) was used to estimate the activity of caspase 3 and caspase 9 in the diluted liver homogenate.

Statistical analysis

Data represented here are the mean values and standard deviation (SD) of three independent experiments, if not otherwise mentioned. Statistical analysis was performed using one-way ANOVA followed by posthoc tests, Student's t-test when a single group was compared against a control group and Dunnett's test when multiple groups (if not otherwise mentioned) were compared against a control group. GraphPad Instat version 3.0 (GraphPad Software, Inc., San Diego, California, USA) was used for analysis.

Chapter 6

Results

Results

Characterization of antisense oligomers

An antisense oligomer was developed to specifically target H-Ras mRNA carrying a uniform point mutation at codon 12 (G-J). This oligomer was synthesized either in its native form or modified by conjugation with a 20-nucleotide FAM-labeled single-stranded antisense sequence (ASO/PS-ASO). Mass spectrometric analysis (Fig. 6.1 A, B) determined that the unlabeled oligomer had a molecular weight of 6604.0 g/mol, whereas the FAM-labeled variant measured 7139.54 g/mol, indicating an increase in mass due to the FAM modification.

Zeta potential measurements using a Zetasizer (Fig. 6.1 C, D) showed a decrease from –24.03 mV to –28.21 mV after FAM conjugation, reflecting a change in surface charge properties. Atomic force microscopy (AFM) data (Fig. 6.1 E, F) indicated that the average height and width of the unmodified PS-ASO were approximately 5.98 nm and 39.7 nm, respectively. Upon FAM labeling, the average height increased to 6.21 nm. This increase in vertical dimension and reduction in surface potential are consistent with prior observations regarding FAM modifications (Shi et al. 2022).

Gel electrophoresis results (Fig. 6.1 G) confirmed that both labeled and unlabeled PS-ASOs had comparable lengths, each measuring approximately 21 nucleotides. Transmission electron microscopy (TEM) imaging (Fig. 6.1 H, I) showed that PS-ASOs formed particle-like structures approximately 40 nm in diameter, composed of globular, drop-shaped subunits with uneven internal density. Each subunit appeared to be interconnected in a spiral arrangement, with visible voids resembling white spots—suggesting a vortex-like architecture within the structure.

Selected area electron diffraction pattern (SAEDP) analysis (Fig. 6.1 J, K) revealed distinct ring formations composed of small, bright spots. These diffraction rings are indicative of polycrystalline materials, as they arise from Bragg reflections generated by numerous discrete crystalline domains. Altogether, these findings support the interpretation that PS-ASOs possess a polycrystalline macromolecular structure.

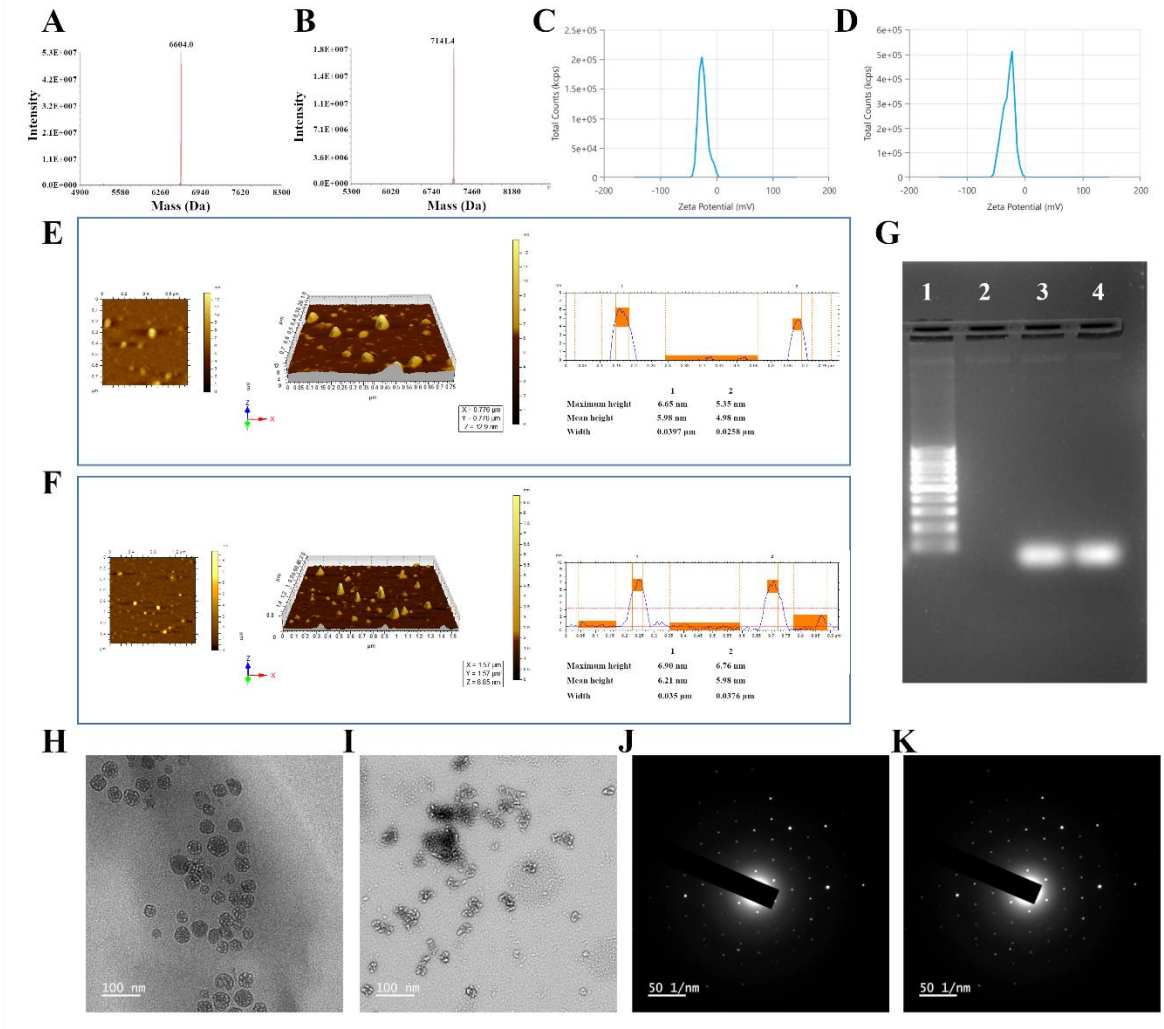

Fig. 6.1. Physicochemical characterization of FAM (fluorescein amidites)-labeled/ unlabeled phophotothoate-backbone modified experimental antisense oligomer (PS-ASO). A. Mass spectrum of unlabeled PS-ASO; B. Mass spectrum of labeled PS-ASO; C. Zeta potential of unlabeled PS-ASO; D. Zeta potential of labeled PS-ASO; E. Atomic force microscopic data of unlabeled PS-ASO; F. Atomic force microscopic data of labeled PS-ASO; G. Gel electrophoresis data of unlabeled PS-ASO (lane 3), labeled PS-ASO (lane 4) and DNA ladder (Lane 1). Data show that both the PS-ASOs were 20 nucleotide-long; H. TEM microphotograph of unlabeled PS-ASO; I. TEM microphotograph of labeled PS-ASO; J. Selected area electron diffraction pattern (SAEDP) of unlabeled PS-ASO; K. SAEDP of labeled PS-ASO.

In Vitro Hemolysis Assessment of PS-ASO

Previous studies have demonstrated the stability of phosphorothioate-modified antisense oligonucleotides (PS-ASOs) in blood (Cunningham et al. 2001; Radi 2019). In the present 12-hour in vitro hemolysis assay conducted under aseptic conditions using whole blood, PS-ASO exhibited minimal hemolytic activity, with hemolysis levels remaining below 3%. In contrast, docetaxel (DTX) showed a slightly higher hemolytic effect of 3.45% (Table 6.1). In vivo animal experiments

In Vivo Evaluation in Animal Model

Hepatic Uptake of PS-ASO

To evaluate hepatic delivery, FAM-labeled PS-ASOs were administered via tail vein injection in healthy rats. Confocal microscopy confirmed the successful accumulation of the labeled oligonucleotides in liver tissue (Fig. 6.2 A–C). Notably, the fluorescence signal persisted up to 8 hours post-injection—the longest time point examined in this study—indicating sustained retention in the liver. The progressive increase in FAM fluorescence over time within hepatic cells suggests that the PS-ASO remains stable and may exhibit an extended biological half-life.

To facilitate quantification of labeled ASOs in circulation, a calibration curve (Fig. 6.1 L) was generated using a spectrofluorimeter by spiking known concentrations of FAM-labeled PS-ASO into rat plasma. This standard curve was used to assess blood concentrations of the labeled oligomers.

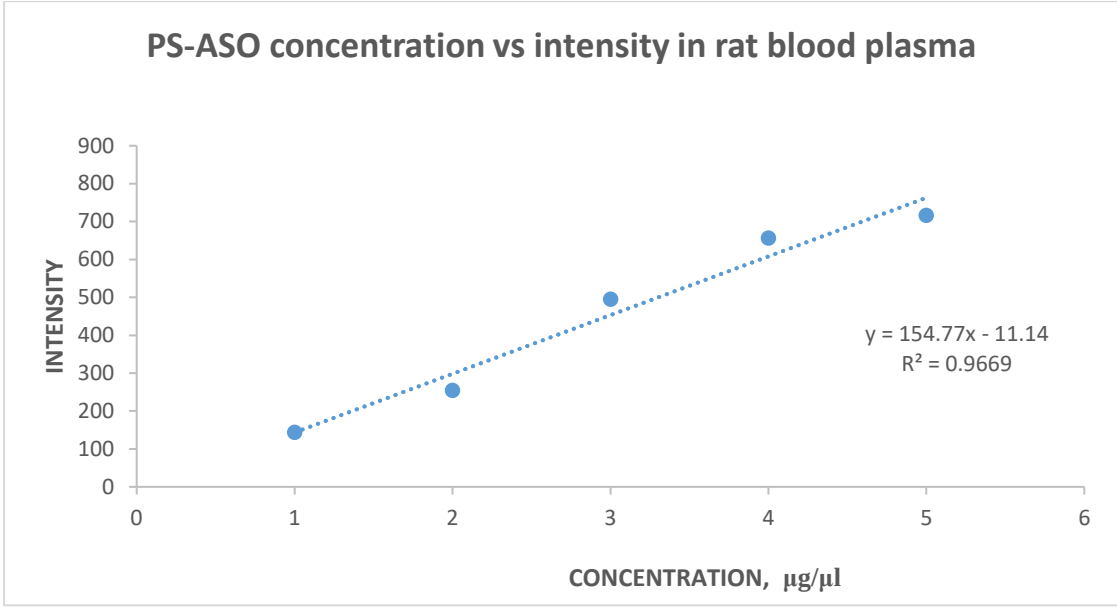

Fig. 6.1 L. Calibration curve of PS-ASO in rat plasma

For the estimation of FAM-labeled PS-ASO from the other tissues (liver, kidney, lung and spleen) (Fig. 6.1 M-P), the known concentrations of the labelled PS-ASO were added to the respective tissue homogenates.

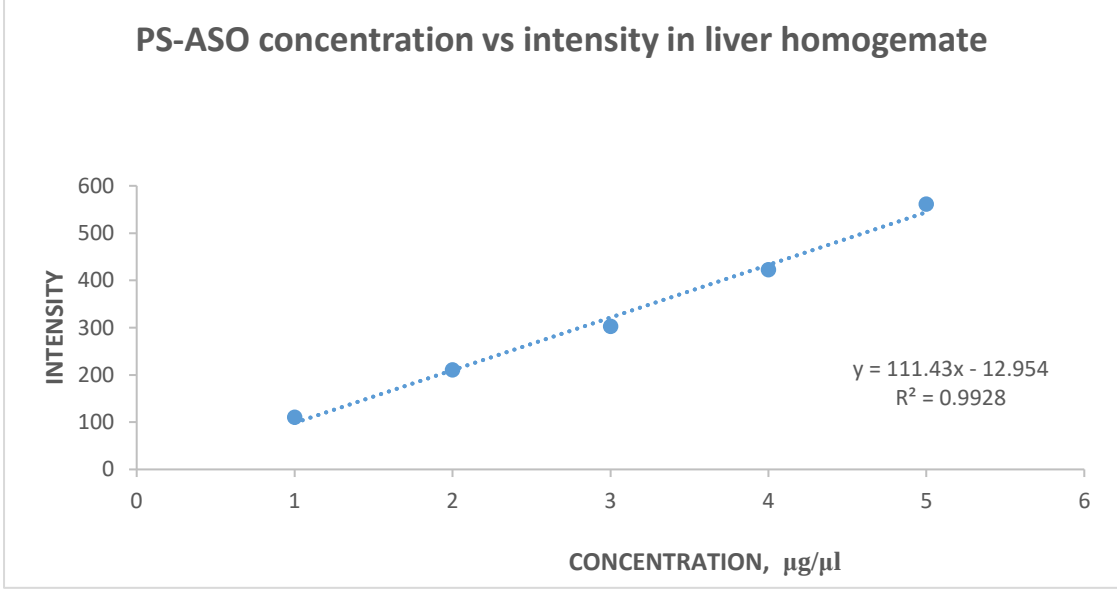

Fig. 6.1 M. Calibration curve of PS-ASO in rat liver homogenate

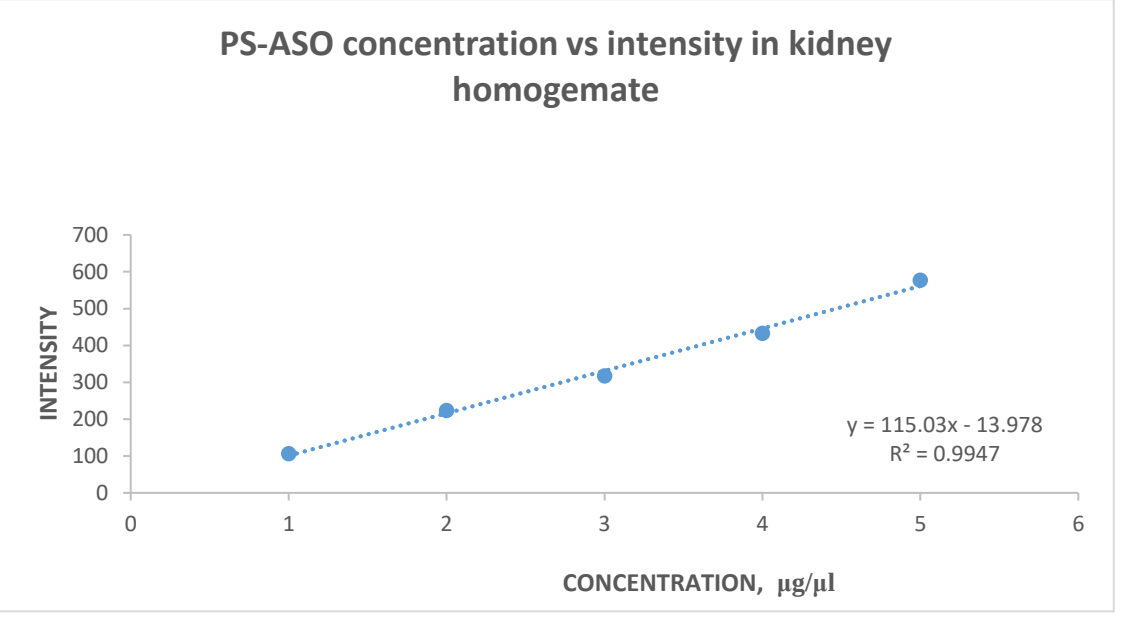

Fig. 6.1 N. Calibration curve of PS-ASO in rat kidney homogenate

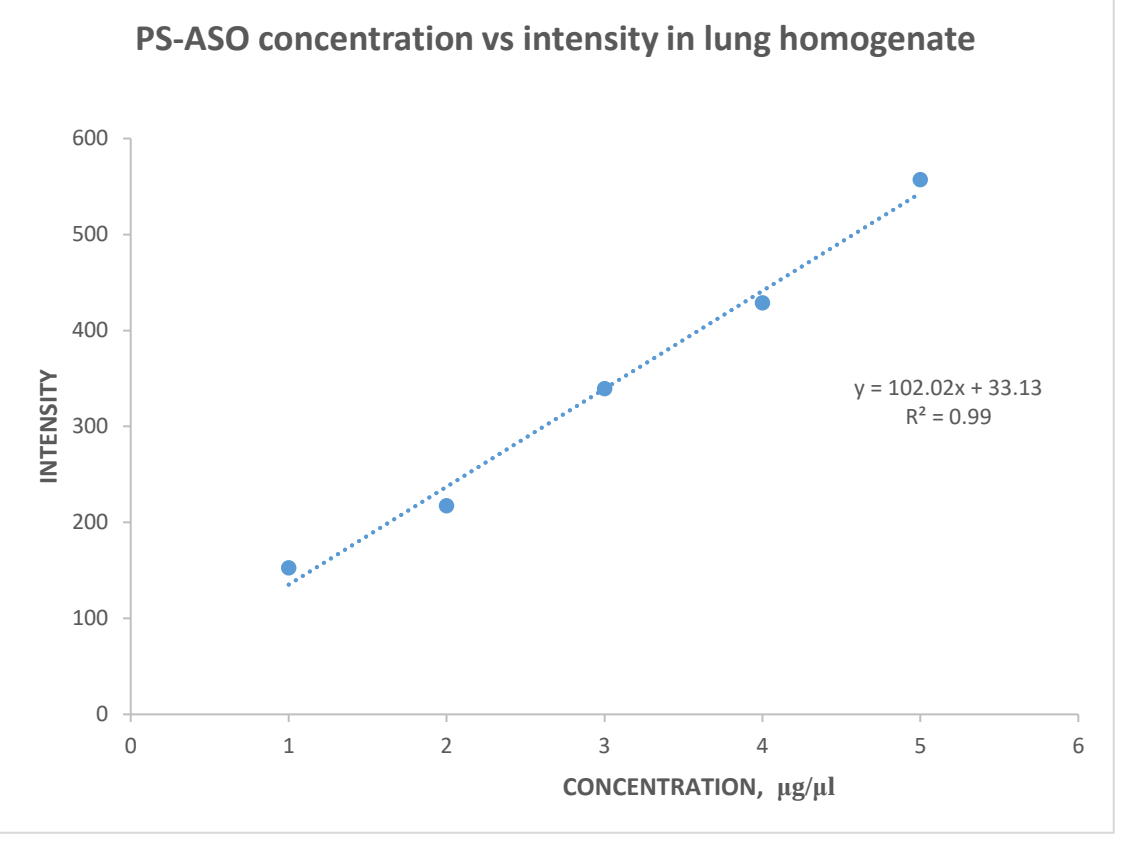

Fig. 6.1 O. Calibration curve of PS-ASO in rat lung homogenate

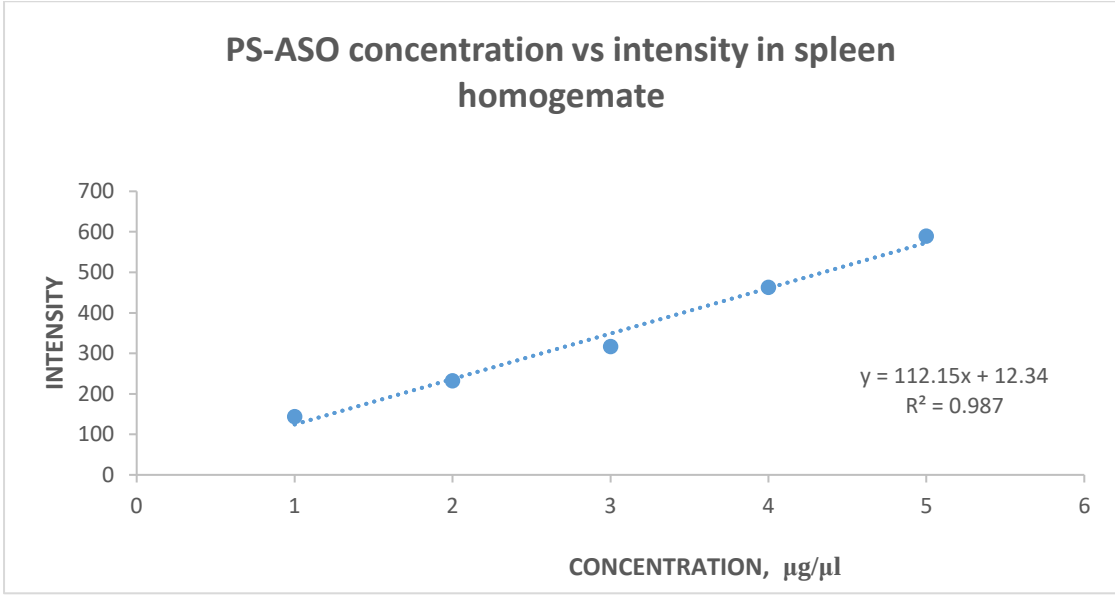

Fig. 6.1 P. Calibration curve of PS-ASO in rat spleen homogenate

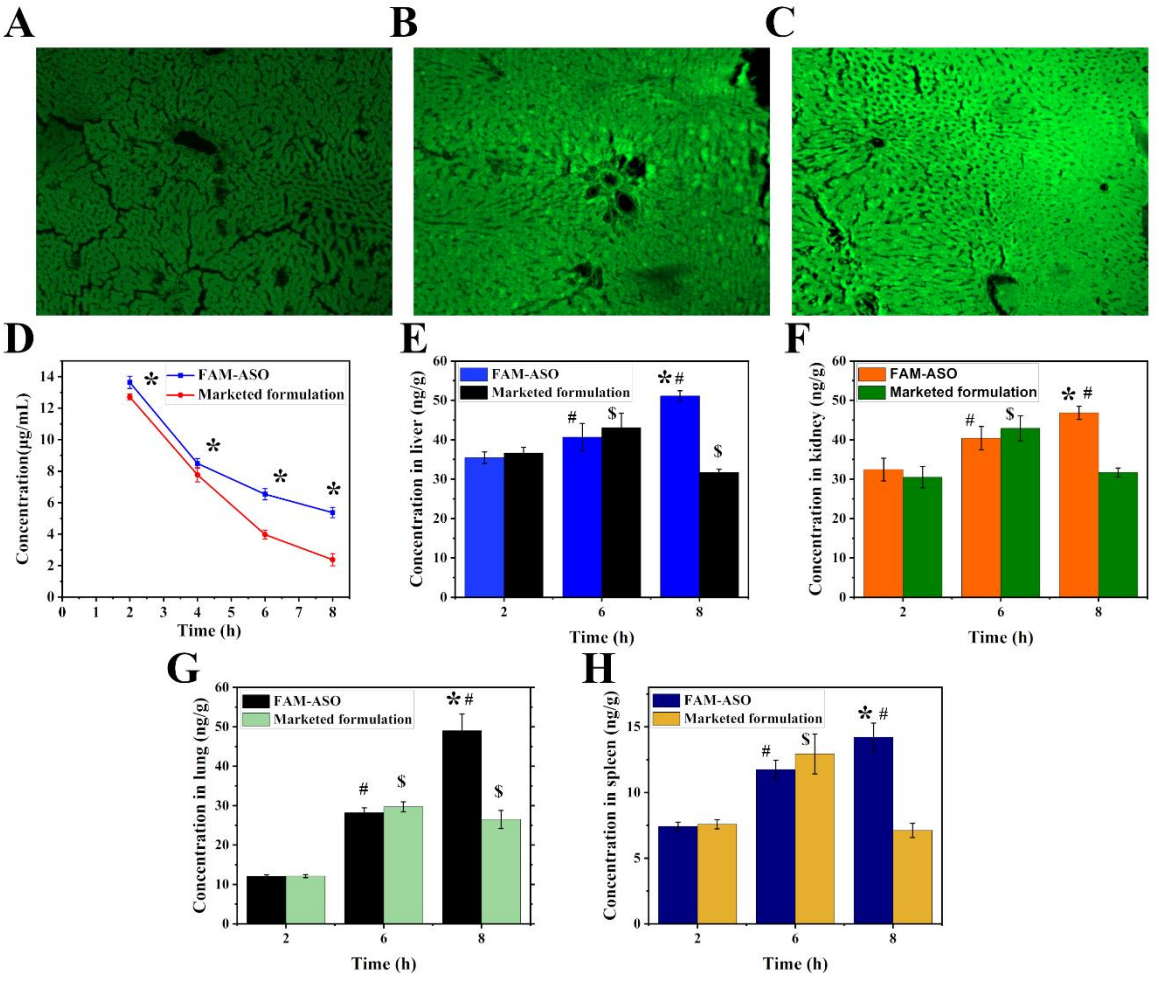

Fig. 6.2. Hepatic uptake, blood level, and few highly perfused tissue distribution patterns of FAM-labeled PS-ASO (FAM-ASO) administered through the tail vein of normal rats. **A**. Confocal microscopic photograph of liver, 2 h after FAM-ASO administration; **B.** Confocal microscopic photograph of liver, 6 h after FAM-ASO administration; **C.** Confocal microscopic photograph of liver, 8 h after FAM-ASO administration; **D.** Blood levels FAM-ASO / docetaxel at different time points after the intravenous administration of FAM-ASO / docetaxel formulation (DXT). Data show mean± standard deviation (n=3); *p< 0.05 is the statistical level of significance when compared against DXT treated blood levels. Data were analyzed using one-way ANOVA followed by Student's t-test. **E.** Liver concentrations of FAM-ASO / docetaxel at the different time points after the intravenous administration of FAM-ASO/ docetaxel formulation (DXT). **F.** Kidney concentrations of FAM-ASO/ docetaxel at the different time points after the intravenous administration of FAM-ASO / docetaxel formulation (DXT). **G.** Lung concentrations of docetaxel at the different time points after the intravenous administration of FAM-ASO / docetaxel formulation (DXT). **H.** Spleen concentrations of

FAM-ASO / docetaxel at the different time points after the intravenous administration of FAM-ASO / docetaxel formulation (DXT).

Data show mean± standard deviation (n=3); *p< 0.05 is the statistical level of significance when compared against DXT treated tissue level at 8 h. $^{\#}$p< 0.05 is the statistical level of significance when compared against FAM-ASO treated tissue level at 2 h. $^{\$}$p< 0.05 is the statistical level of significance when compared against DXT treated tissue level at 2 h. Data were analyzed using one-way ANOVA followed by Student's t-test for the data marked with (*) at 8 h compared against respective DXT concentrations, and by Dunnett's t-test for the comparison with the data (marked with $, #) of FAM-ASO/ DXT against their values at 2 h.

Pharmacokinetic and Biodistribution Study

A safe dose of 2 mg/kg body weight of unlabeled PS-ASO—or the equivalent amount of the FAM-labeled variant—was selected for this study. Subsequently, pharmacokinetic profiling and biodistribution analysis were performed using FAM-labeled PS-ASO administered intravenously via the tail vein in rats. As previously reported by Cunningham et al. (2001) and Radi (2019), PS-ASOs exhibit high stability in the bloodstream. For comparison, blood concentrations and tissue distribution of docetaxel (DTX), delivered as Taxotere®, were also evaluated under the same conditions (Fig. 6.2).

A spectrofluorimeter was employed to quantify the levels of FAM-labeled PS-ASO in blood and selected tissues. From these data, key pharmacokinetic parameters were calculated. Compared to DTX, PS-ASO exhibited a more gradual decline in blood concentration over the observation period (2 to 8 hours), indicating slower systemic clearance (Fig. 6.2 D). This prolonged circulation time suggests that PS-ASO may offer a more sustained therapeutic window.

The half-life values for PS-ASO and DTX were estimated at 6.4 and 4.8 hours, respectively. The area under the curve ($AUC_{0–8}$), representing bioavailability over the first 8 hours, was 67.5 µg·h/mL for DTX and 84.2 µg·h/mL for PS-ASO. Corresponding mean body clearance rates were 0.0059 L/h for DTX and 0.0047 L/h for PS-ASO (Table 6.1), as described by Mukherjee (2022). Collectively, these findings indicate that PS-ASO exhibits favorable pharmacokinetic properties with extended blood retention compared to DTX.

Table 6.1: In vitro haemolysis and in vivo pharmacokinetic profile of the normal rats received experimental treatments.

Treatment	$t_{1/2}$ (h)[a]	AUC_{0-t} ($\mu g.h.ml^{-1}$)	CL($L.h^{-1}.kg$)	Haemolysis (%)
FAM-ASO (FAM-labeled PS-ASO)	6.4±0.13*	84.2±2.18*	0.0047±0.000*	2.8±0.03*
DXT (taxotere®)	4.8±0.18	67.5±2.56	0.0059±0.0002	3.45±0.02

Note: [a]Data show mean± SD (standard deviation) (n=3).
Abbreviations: $t_{1/2}$, half-life; AUC_{0-t}, the area under the plasma drug concentration–time curve from the time of injection to a determined time point at 8 h; CL, operative body clearance.

*p<0.05 was considered statistically significant difference when FAM-ASO data were compared to the data of DXT-treated group of rats.

Tissue Distribution of PS-ASO

The distribution of PS-ASO across various tissues showed distinct patterns over time (Fig. 6.2 E–H). Within the first two hours post-injection, PS-ASO levels were predominantly concentrated in the liver and kidneys. These concentrations further increased at 6 and 8 hours, the final time points of the study. In contrast, PS-ASO levels in the lungs and spleen were initially low at 2 hours. However, a marked increase in lung concentrations was observed at 6 and 8 hours, with lung levels nearly matching those of the liver and kidneys by the 8-hour mark. At this time point, tissue distribution followed the descending order: liver > lungs > kidneys.

To validate these findings, tissue samples were also analyzed using confocal microscopy (Fig. 6.3), which confirmed the spectrofluorimetric data. Notably, the spleen exhibited minimal accumulation of PS-ASO, consistent with the quantitative analysis. For comparison, tissue

distribution of DTX following intravenous administration of Taxotere® was also evaluated. DTX concentrations peaked at 6 hours and declined thereafter across the assessed tissues.

These observations highlight the distinct biodistribution profiles of PS-ASO and DTX, with PS-ASO demonstrating prolonged retention and a different tissue accumulation pattern compared to the conventional chemotherapeutic agent.

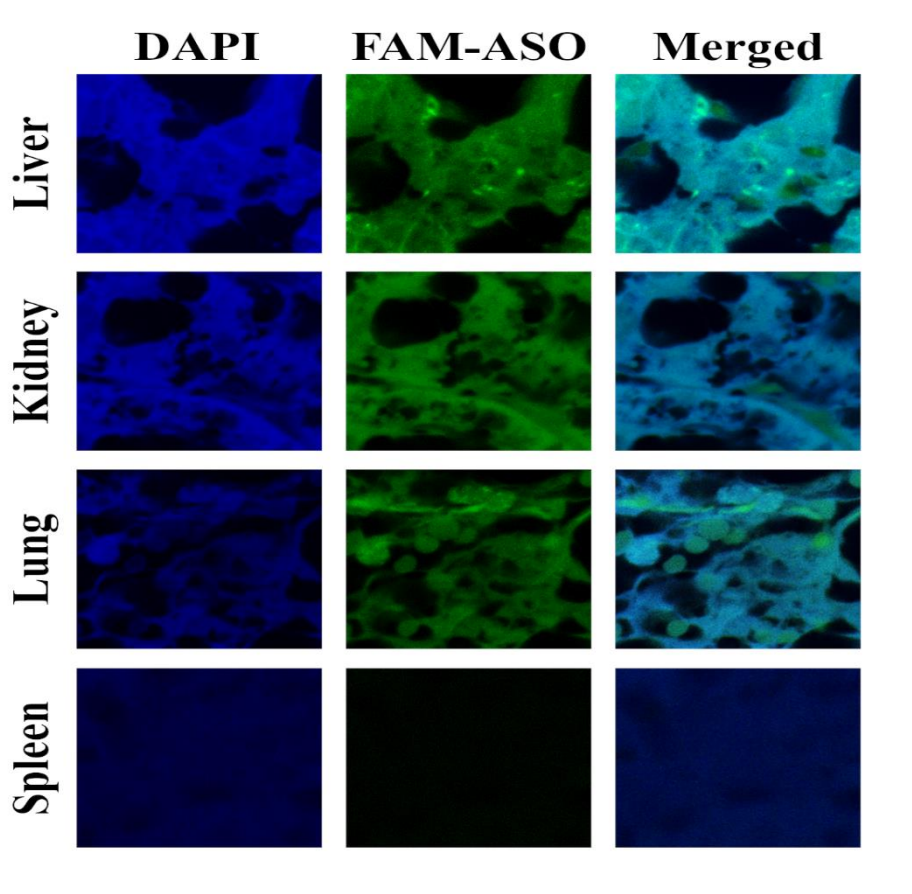

Fig. 6.3. Confocal microscopic photograph (60x) of some highly perfused organs at 8 h after the FAM-labeled PS-ASO (FAM-ASO) administration through the tail vein of normal rats. Counter-stained with DAPI showed blue color, FAM gave green fluorescence.

Impact of PS-ASO Treatment on Perfused Organs in Healthy Rats

The potential effects of PS-ASO and DTX on major highly perfused organs—namely the liver, kidneys, lungs, and spleen—were evaluated in normal rats. Histological examination revealed

that DTX administration induced several morphological alterations and signs of toxicity. In the liver, DTX-treated rats displayed widened sinusoidal spaces (Fig. 6.4), while lung sections showed significant thickening of the interalveolar septa, indicating pulmonary stress.

In contrast, no histopathological abnormalities were observed in rats treated with PS-ASO for either three or six weeks, with organ structures remaining comparable to those of untreated controls. Renal tissues from the DTX group demonstrated clear signs of nephrotoxicity, including glomerular shrinkage, tubular dilation, interstitial fibrosis, and expansion of Bowman's capsule.

These findings suggest that, unlike DTX, PS-ASO administration did not result in detectable morphological damage or toxic effects in the assessed organs over the course of the study.

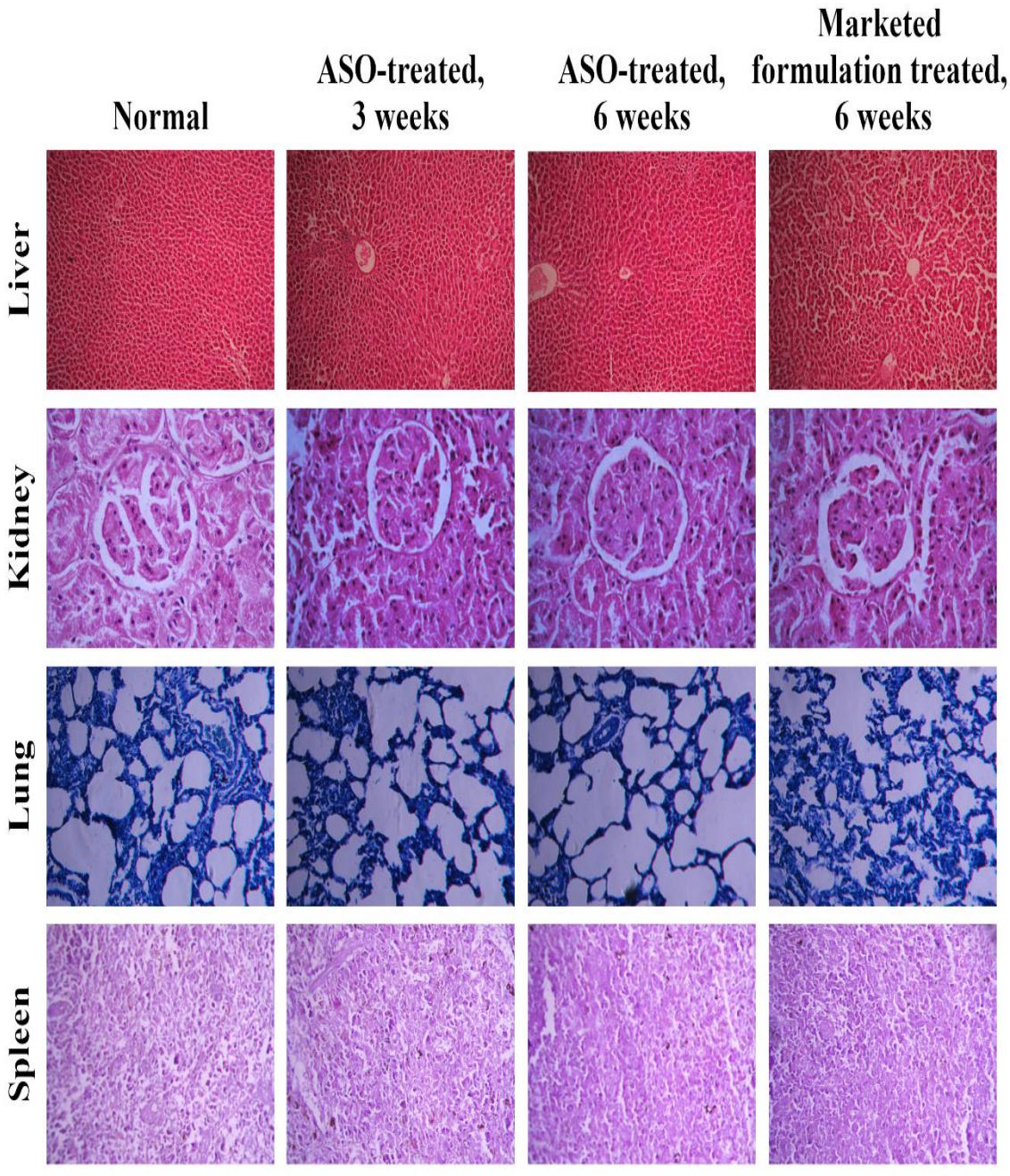

Fig. 6.4. Histological microscopic photographs of liver (10x), kidney (40x), lung (40x) and spleen (40x) of normal (untreated) rats and normal rats received experimental treatments.

Antineoplastic in vivo study

Hepatic macroscopic examination

Macroscopic Observations of Tumor Development and Treatment Effects gross examination revealed distinctive tumor formations in rats from the carcinogen control group (Fig. 6.5 A–D). These animals exhibited prominent grey-white hyperplastic nodules (HNs) that progressed into large hepatocellular carcinoma (HCC) masses, transitioning from grey HNs to reddish tumor lesions. In some cases, aggressive tumor growth led to rupture through the liver surface, forming visible protrusions (Fig. 6.5 A). Furthermore, metastatic lesions were observed in the lungs of two out of six rats in this group, indicating hepatic metastasis (Fig. 6.5 E).

In contrast, rats receiving PS-ASO therapy for three weeks displayed fewer and smaller grey-white nodules (Fig. 6.5 B). A notable 66% reduction in tumor incidence was recorded in this group. After six weeks of PS-ASO administration, hyperplastic nodules had completely regressed in the livers of treated animals. Metastatic spread to the lungs, as seen in the control group, was absent in PS-ASO-treated rats.

Taxotere® treatment also contributed to a reduction in tumor size; however, the number of tumor nodules remained higher in the Taxotere® group compared to those treated with PS-ASO for both three and six weeks (Table 6.2).

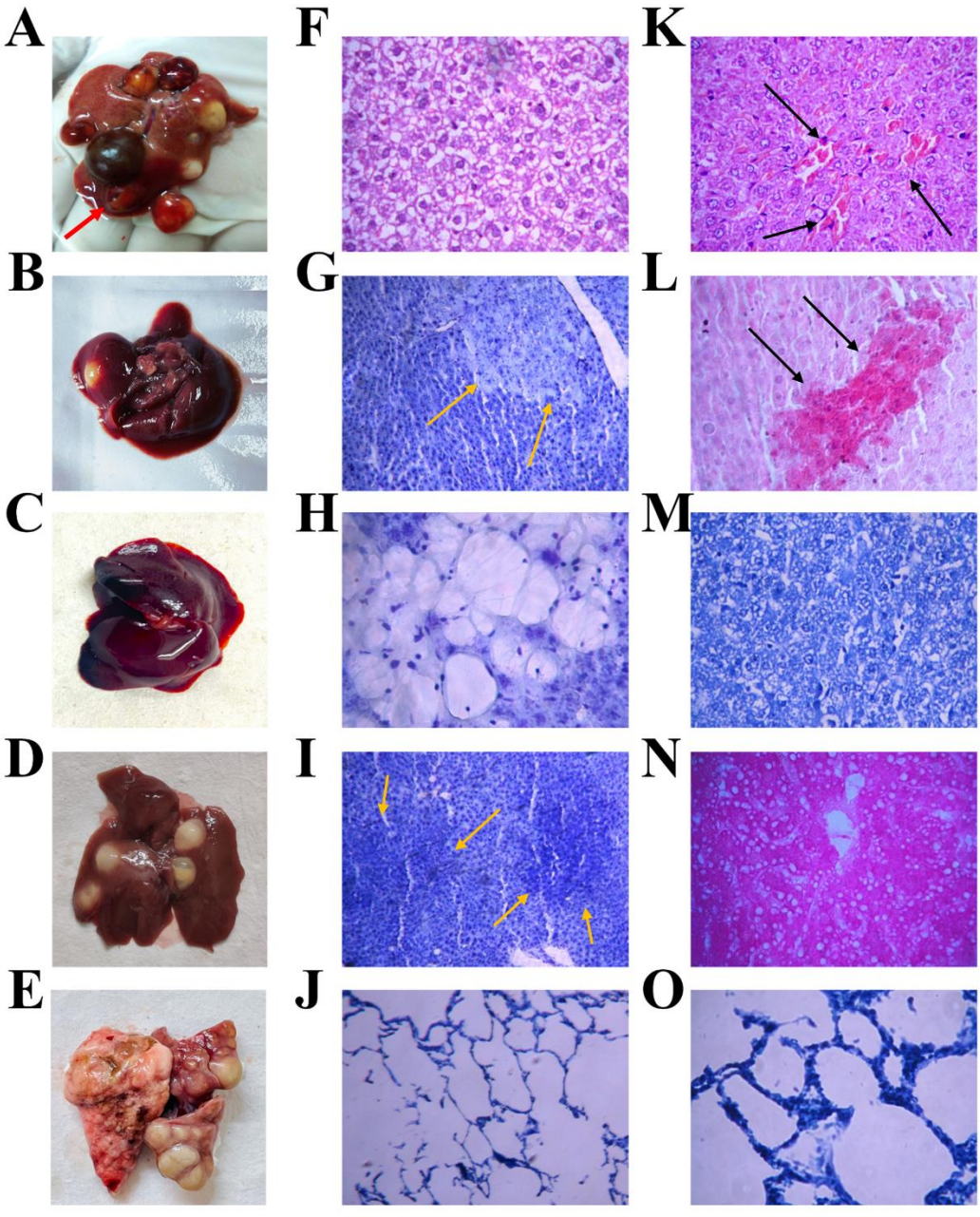

Fig. 6.5. Macroscopic and microscopic liver and metastatic lung images of rats treated with carcinogen and received the experimental treatments. PS-ASO here also represented as ASO in the figure. **A.** A macroscopic liver image of carcinogen-induced HCC rats. The photograph shows multiple tumors and grey-white hyperplastic tumor nodules, and a tumor bulged out, cracking the liver surface (shown by a red arrow head). **B.** A macroscopic liver image of carcinogen-induced HCC rats received PS-ASO-treatment for 3-weeks. **C.** A macroscopic liver image of carcinogen-induced HCC rats received PS-ASO-treatment for 6-weeks. **D.** A

macroscopic liver image of carcinogen-induced HCC rats received docetaxel formulation (DXT) for 6-weeks. **E.** A macroscopic image of carcinogen-induced HCC rat lung, showing metastatic tumor development. Various preneoplastic and neoplastic hepatic lesion images (F-I, K-N) (40x) and microscopic lung images (J and O) (60x) in carcinogen-induced HCC rats. **F.** Ground glass lesion; **G.** Mixed cell focal lesion (shown by arrow heads), upon PAS staining; **H.** Spongiosis hepatis, showing distended hepatic stellate cells, upon PAS staining; **I.** Darker blue basophilic lesions (shown by arrow heads), upon TB staining; **J.** Alveoli structure in tumor area, showing emphysema, upon TB staining; **K.** Angiectasis lesion (Peliosis hepatis), with increased sinusoidal space and filled with blood cells (shown by arrow heads), upon HandE staining; **L.** Hypereosinophilic lesion (shown by arrow heads), upon HandE staining; **M.** Carcinoma in the hepatocytes, upon TB staining; **N.** Fatty liver, where fat droplets appeared as clear-hollow in the hepatocyte upon HandE staining; **O.** Alveoli structure in non-tumor area, upon TB staining.

HandE, Hematoxylin-eosin, TB, toluidine blue, PAS, periodic acid-Schiff

Microscopic Evaluation of Hepatic Lesions

Histological examination of liver tissues from carcinogen control rats revealed widespread hepatic altered foci (HAFs) scattered throughout the non-tumorous regions. These animals displayed extensive tumor infiltration, with a high prevalence of various preneoplastic lesions. Notable lesion types included ground-glass foci, mixed cell foci, basophilic lesions, spongiosis hepatis, angiectatic changes, and hypereosinophilic (pre-apoptotic) areas (Fig. 6.5 F–N).

Among the early preneoplastic alterations, ground-glass lesions—rich in glycogen—were observed to transition into mixed cell foci, which exhibited diminished glycogen and increased ribosomal content (Fig. 6.5 F–G). These lesion types were strongly associated with hepatocellular carcinoma (HCC) development. Ribosome-dense basophilic lesions (Fig. 6.5 I) and hypertrophied hepatic stellate cells (Fig. 6.6 H) contributed to the formation of spongiosis hepatis, a hallmark of progressive hepatic degeneration leading to malignancy in the control group.

Lesion size varied considerably depending on the treatment regimen. PS-ASO administration for six weeks led to an increased number of small preneoplastic foci (<1 mm) and a reduction in large lesions (>3 mm), indicating a therapeutic effect (Table 6.1). Enlarged hepatic stellate cells—often associated with spongiosis hepatis and showing a multilocular cystic structure with flocculent eosinophilic cytoplasm—were prominent in untreated carcinogen-exposed livers and are typical of liver undergoing malignant transformation.

Pulmonary metastatic nodules showed signs of emphysema in some rats (Fig. 6.5 J). Angiectatic lesions (meliosis hepatis) were also noted in the control group; these presented as dilated sinusoids congested with blood and surrounded by atrophic hepatocytes (Fig. 6.5 K), further supporting their role in HCC pathogenesis.

Apoptotic hepatocytes, identified by hypereosinophilic cytoplasm and condensed, fragmented nuclear material, were rarely seen in control rats but became more evident following six weeks of PS-ASO treatment. Hallmarks of apoptosis—such as nuclear blebbing, chromatin condensation, apoptotic body formation, and the presence of multinucleated or polypoid hepatocytes—were observed (Fig. 6.5 L). These regions, enriched in apoptotic cells, formed distinctive hypereosinophilic foci.

The incidence of overt hepatic tumors was highest in the carcinogen control group (Fig. 6.5 M). Degenerating hepatocytes often showed cytoplasmic vacuolization or hollowing due to shrinkage or fragmentation. Fat accumulation was another common observation, with hepatocyte cytoplasm displaying vacuoles characteristic of lipid buildup, as evident in HandE-stained sections (Fig. 6.5 N).

In summary, PS-ASO treatment significantly reduced both the number and size of hepatic altered foci in carcinogen-treated rats, with the most pronounced effect observed after six weeks of therapy compared to three weeks (Table 6.2).

Table 6.2. Tumor incidences, and sizes of hepatic altered focal lesions (HAFs) in experimental rats

Animal groups	**Tumor incidences (out of six animals in each group)**	**Incidences of (%) hepatic altered focal lesions (HAF)/cm^2 hepatic tissue area** **< 1 mm** **1 mm-3 mm** **>3mm**
Group A (normal control)	0/6	
Group B (carcinogen control)	6/6	15±2.1 32±2.8* 53±3.8*
Group C (carcinogen-treated rats treated with ASO for 3 weeks)	2/6	46±2.6$^{\#}$ 33±2.2*,$^{\#}$ 21±1.8*,$^{\#}$
Group D (carcinogen-treated rats treated with ASO for 6 weeks)	0/6	81±3.4$^{\#}$ 14±2.0*,$^{\#}$ 05±1.2*,$^{\#}$
Group E (carcinogen-treated rats treated with Taxotere® for 6 weeks)	4/6	32±1.8$^{\#}$ 38±2.2*,$^{\#}$ 30±1.4*,$^{\#}$
Group F (normal rats treated with ASO, as ASO control)	0/6	

Data show mean±SD (n = 6); Data were analyzed by two way ANOVA, followed by Dunnett's test.

*$p < 0.05$, when alteration of HAF incidences was compared with those of the size (< 1 mm)

$^{\#}p < 0.05$, when alteration of HAF incidences was compared with those of Group B rats

Effect of PS-ASO on Hepatic Altered Foci and Lung Metastases

In carcinogen-exposed rats, a six-week course of PS-ASO treatment produced the most substantial reduction in both the size and number of hepatic altered foci (HAFs) compared to

the three-week treatment. Notably, the six-week PS-ASO group exhibited the lowest incidence (10%) of large HAFs (>3 mm) and the highest frequency of small foci (<1 mm), suggesting a marked delay in tumor progression. Conversely, the carcinogen control group displayed the highest occurrence (53%) of large HAFs and the fewest small lesions, reflecting aggressive disease development. In normal rats treated with PS-ASO, hepatic tissue architecture remained intact even after six weeks, indicating no apparent toxicity.

In rats treated with Taxotere®, the proportion of large HAFs was greater than that observed in the PS-ASO-treated groups but still lower than in untreated carcinogen-exposed animals. These rats also showed a reduced number of small HAFs compared to PS-ASO-treated rats, although still more than in the carcinogen control group. The distribution of intermediate-sized lesions (1–3 mm) in Taxotere®-treated rats mirrored the trend seen for small lesions.

As previously described, hepatic tumors in some carcinogen control rats metastasized to the lungs (Fig. 6.5 E). In these cases, histological examination revealed emphysematous changes in the lung tissue, characterized by the destruction of alveolar walls and the formation of abnormally large air spaces (Fig. 6.5 J). Normal-appearing alveolar wall thickness was observed in non-tumorous regions of the lung (Fig. 6.5 O).

In Situ Hybridization for H-Ras mRNA Expression

To assess gene silencing at the cellular level, in situ hybridization using digoxigenin-labeled PS-ASOs was performed. This analysis revealed that PS-ASO treatment significantly downregulated H-Ras mRNA expression, which was markedly elevated in the livers of carcinogen control rats (Fig. 6.6 A; Table 6.3). Quantification was based on established semi-quantitative scoring systems for gene and protein expression (Meyerholz and Beck 2018; Fedchenko and Reifenrath 2014; Rizzardi et al. 2012).

Importantly, suppression of *H-Ras* expression was more pronounced in rats treated with PS-ASO for six weeks compared to those treated for three weeks. These results confirm that the specific antisense sequence effectively inhibited the expression of the mutant *H-Ras* gene at the transcript level.

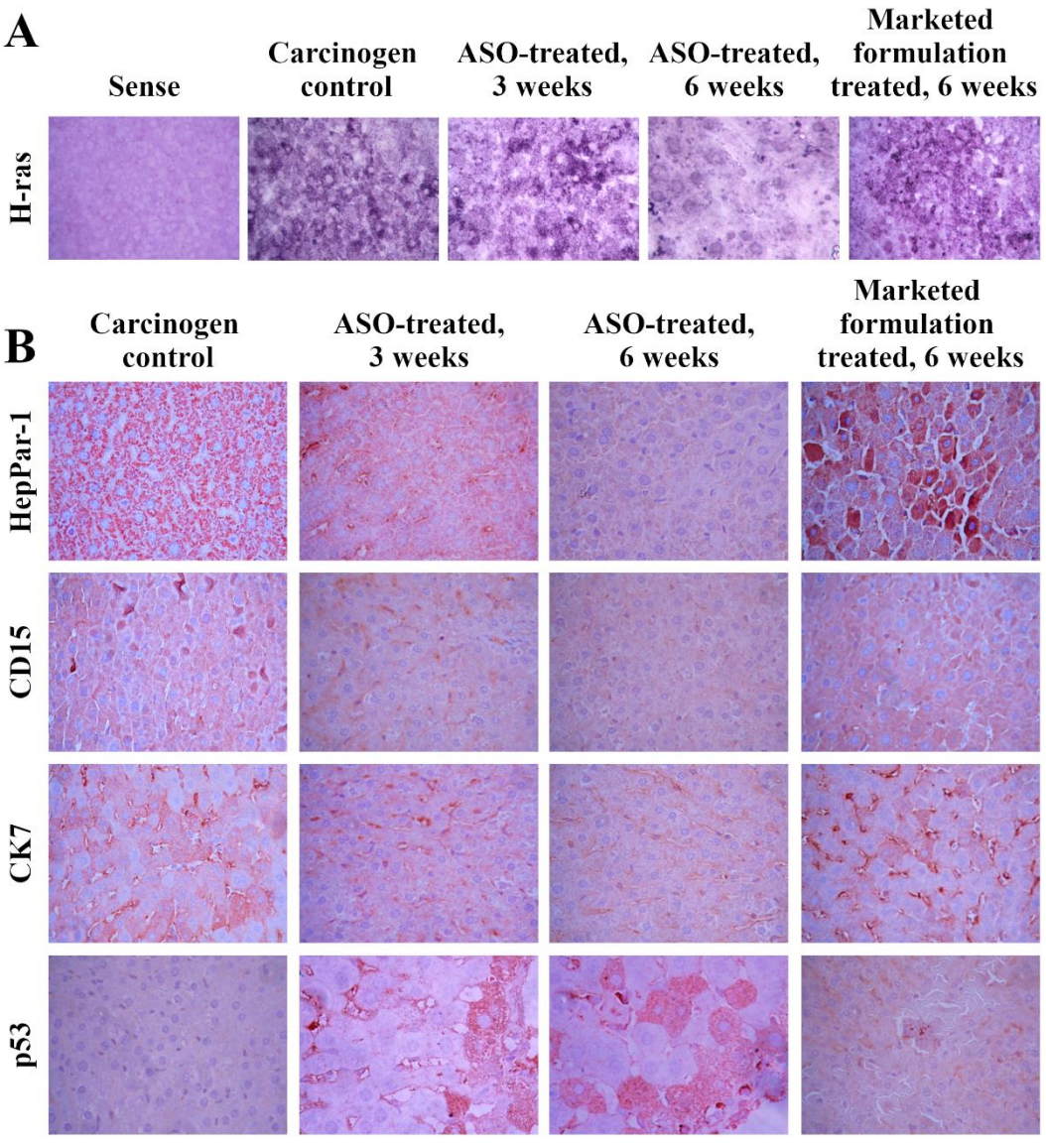

Fig. 6.6. *H-Ras* gene expression patterns (assessed by in situ hybridization) and various HCC marker protein expressions (determined by immunohistochemical method) in the liver of the experimental rats treated with carcinogen and received the experimental treatments (PS-ASO here also represented as ASO in the figure). **A.** Photographs (40x) show sense control, carcinogen-induced HCC rats (carcinogen control), and carcinogen-induced HCC rats treated with various experimental treatments. B. Photographs (40x) of Hep par I, CD-15, CK-7 and p53 protein expression patterns in carcinogen control rats and carcinogen-induced HCC rats received various experimental treatments.

The absence of mutated *H-Ras* gene expression in the sense control data suggests that PS-ASO is effective at blocking the expression of the mutated *H-Ras* gene. The hepatic tissues of the rats exposed with the carcinogen exhibited a moderate decrease in *H-Ras* gene expression following the injection of Taxotere®.

Table 6.3

Combined semi-quantitative scoring analysis of data of *H-Ras* gene expression by in-situ hybridization, and hep-par-I, CD-15, CK-7 and p53 protein analysis by immunohistochemistry in experimental rats

Treatment received/ Expression type	*H-Ras* expression [a](Combined semi-quantitative scoring)	Hep-Par-I protein expression (Combined semi-quantitative scoring)	CD-15 protein expression (Combined semi-quantitative scoring)	CK-7 protein expression (Combined semi-quantitative scoring)	P^{53} protein expression (Combined semi-quantitative scoring)
Carcinogen control rats	95.5 ±2.3	98.2 ±3.4	60.8 ±2.2	52.8 ±2.5	1.1 ±0.04
Carcinogen-treated rats received PS-ASO treatment for 3-weeks	76.2 ±4.2*	60.5 ±2.6*	27.4 ±1.8*	30.4 ±3.0*	25.4 ±1.4*
Carcinogen-treated rats received PS-ASO treatment for 6-weeks	27.3 ±1.2*	28.8 ±1.7*	14.6 ±1.3*	22.8 ±1.8*	36.8 ±2.3*
Carcinogen-treated rats received Docetaxel treatment for 6-weeks	85.8 ±3.8*	80.2 ±4.1*	25.6 ±3.2*	34.5 ±2.6*	18.7 ±1.4*

[a]Combined semi-quantitative scoring was done based on staining intensity and % of stained cells/cm^2 area. Data show mean ± standard deviation and were analyzed based on 25 slides (25 x4 observations) in each case.

*$p<0.05$, when data were compared with respect to carcinogen control, assessed by one-way ANOVA followed by Dunnett *t*-test

Immunohistochemical Analysis

Immunomarkers are widely recognized for their ability to differentiate well-differentiated from poorly differentiated hepatocellular carcinoma (HCC). For this study, we selected several commonly used HCC immunohistochemical markers: Hep par-1, CK-7, CD-15, and p53. Immunohistochemical staining was performed to assess Hep par-1 protein expression in liver tissue sections (Fig. 6.6 B). The carcinogen-control group exhibited elevated Hep par-1 expression, which was notably reduced following PS-ASO treatment. The greatest decline in Hep par-1 levels was observed after six weeks of PS-ASO therapy (Table 6.3). Normal control rats and those treated with PS-ASO alone showed no detectable Hep par-1 expression. Hepatic Hep par-1 expression in carcinogen-exposed rats treated with Taxotere® was similar to that in rats receiving three weeks of PS-ASO treatment.

Markers CK-7 and CD-15 were prominently expressed in carcinogen-treated rats. Both PS-ASO and Taxotere® treatments reduced CK-7 and CD-15 protein expression, with six weeks of PS-ASO therapy demonstrating the most significant suppression compared to three weeks of PS-ASO or Taxotere® treatment (Fig. 6.6 B).

In contrast, p53 protein expression was minimal in carcinogen control rats (Fig. 6.6 B). Treatments elevated p53 levels in the following order: six-week PS-ASO > three-week PS-ASO > Taxotere®, indicating a treatment-related increase in p53 expression from low to high.

Scanning Electron Microscopy (SEM) Evaluation

SEM images (Fig. 6.7) detailed the ultrastructural characteristics of liver tissues from experimental rats, with some images shown at higher magnifications for clarity. The normal control liver ultrastructure (Fig. 6.7 A, B) revealed tightly packed arrays of small cuboidal and polygonal hepatocytes (Russo et al. 1977). Although the cell surfaces were not perfectly smooth, well-defined sinusoidal gaps were evident, along with distinct canals for arteries, portal veins, and the central vein. Hepatocytes were generally uniform in size.

In carcinogen-control rats (Fig. 6.7 C, D), the hepatocytes appeared more heterogeneous and densely packed, with numerous small, round, and newly formed cells. Sinusoidal gaps were markedly reduced and often filled with cellular debris and necrotic material. The central vein, portal artery, and hepato-portal canals appeared constricted. Malignant cells exhibited enlarged and more numerous microvilli on their surfaces.

In rats treated with PS-ASO for three weeks, the hepatic ultrastructure (Fig. 6.7 E, F) showed some improvement: hepatocytes maintained a dense arrangement but exhibited a more rounded or oval shape in areas. Sinusoidal gaps were visible in several regions, although cells remained closely associated, resembling malignant morphology.

Following six weeks of PS-ASO treatment, the hepatic ultrastructure (Fig. 6.7 G, H) more closely resembled normal liver architecture. Hepatocytes appeared cuboidal or hexagonal with rough cell surfaces typical of healthy liver cells (Fig. 6.7 F, G).

By contrast, Taxotere® treatment did not produce notable ultrastructural improvements in carcinogen-exposed rats (Fig. 6.7 I, J). Hepatocytes remained heterogeneous and densely packed, with disorganized sinusoidal spaces and numerous newly formed small cells, indicative of persistent hepatic damage.

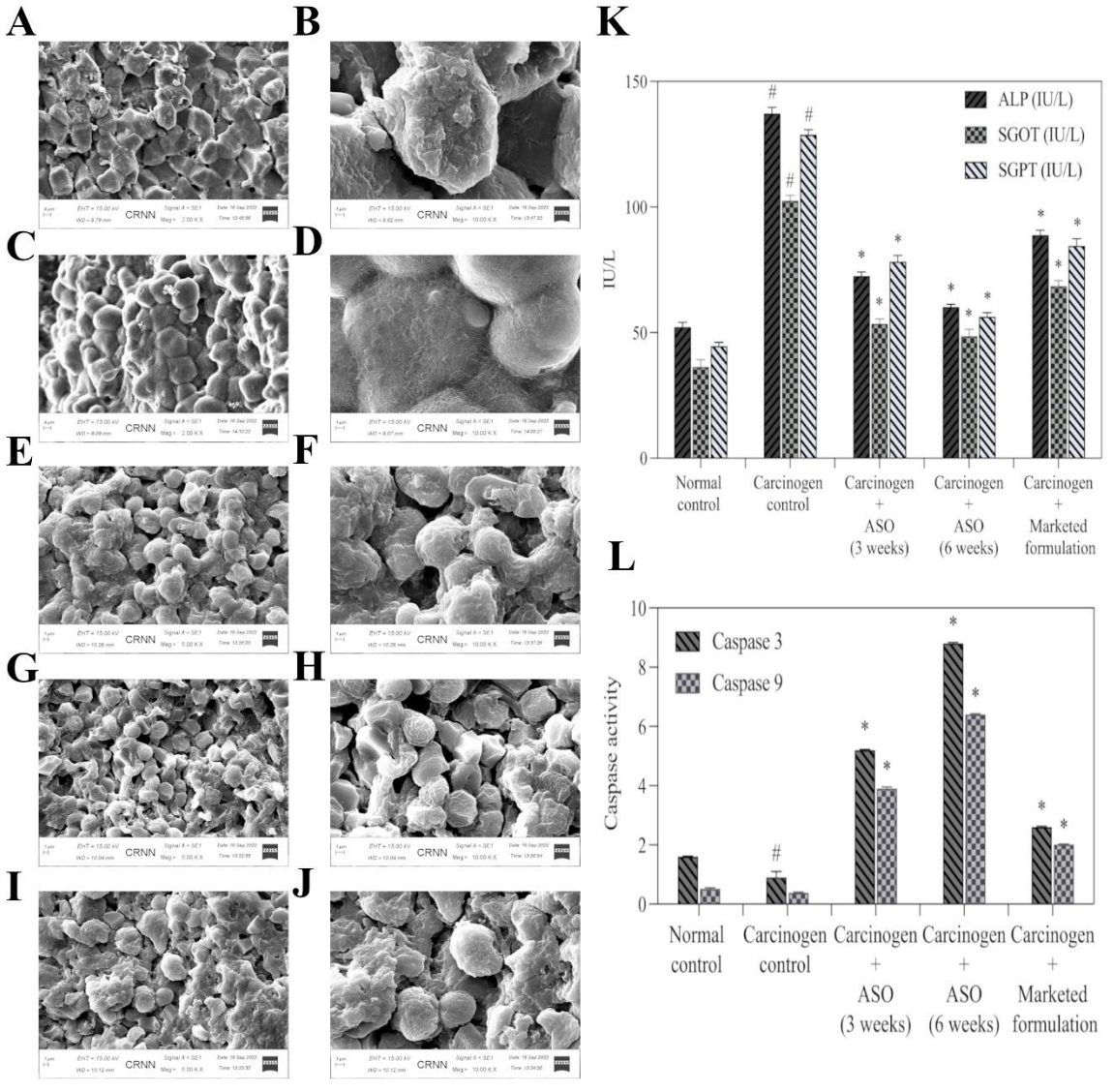

Fig. 6.7. Scanning electron microscopic images of hepatic tissues, and blood levels of hepatic marker enzymes, and hepatic caspase 3/9 activities of various control and treated experimental rats. PS-ASO is here also represented as ASO in the figure. A. Hepatic tissue image of normal (untreated) rats (2,000x); B. Portion of the image 7. A, at higher magnification (10,000x); C. Hepatic tissue image of carcinogen-induced HCC rats (2,000x); D. Portion of the image 7. C, at higher magnification (10,000x); E. Hepatic tissue image of carcinogen-induced HCC rats treated with PS-ASO for 3-weeks (5,000x); F. Portion of the image 7. E, at higher magnification (10,000x); G. Hepatic tissue image of carcinogen-induced HCC rats treated with PS-ASO for 6-weeks (5,000x); H. Portion of the image 7. G, at higher magnification (10,000x); I. Hepatic tissue image of carcinogen-induced HCC rats treated with docetaxel formulation for 6-weeks (5,000x); J. Portion of the image 7. I, at higher magnification (10,000x); K. ALP, SGOT and

SGPT blood levels of various control and treated experimental rats. L. Hepatic caspase 3/9 activities of various control and treated experimental rats.

Caspase-3/9 activities are expressed as the fold change in enzyme activity over carcinogen control data obtained from hepatic tissue homogenates.

Data (Fig. 6.7. K and 7. L) show mean± standard deviation (n=3); *p< 0.05 is the statistical level of significance when the data of carcinogen treated rats received various treatments were compared against carcinogen control HCC rats. $^{\#}p< 0.05$ is the statistical level of significance when compared the data of carcinogen control HCC rats against those of normal (untreated) rats. Data were analyzed using one-way ANOVA followed by Student's t-test for the data of carcinogen control HCC rats and compared against normal (untreated) control rats, and by Dunnett's t-test for the comparison of data of rats received experimental treatments to the data of carcinogen control HCC rats.

Effect of PS-ASO/DTX on ALK, SGPT, and SGOT Activities in Experimental Rat Livers

Several clinical indicators reflect liver function abnormalities, with the liver being the primary metabolic organ. Among these, serum levels of alkaline phosphatase (ALK), serum glutamate-pyruvate transaminase (SGPT), and serum glutamate-oxaloacetate transaminase (SGOT) serve as crucial biochemical markers for assessing liver health and function (Mukherjee et al. 2022). In the carcinogen-control rats, the levels of ALK, SGPT, and SGOT were significantly elevated. Treatment with PS-ASO/DTX in carcinogen-exposed rats led to a marked reduction in these enzyme levels (Fig. 6.7 K). Notably, after six weeks of PS-ASO therapy, enzyme activities approached normal values, suggesting an improvement in hepatic function mediated by PS-ASO.

Effect of PS-ASO/DTX on Caspase-3 and Caspase-9 Activities in Experimental Rat Liver

Activation of caspase-3 and caspase-9 is a well-known mechanism driving apoptosis in cancer cells (Brentnall et al. 2013). In the livers of carcinogen-treated rats, six weeks of PS-ASO treatment induced a more pronounced increase in caspase-3 and caspase-9 activities compared

to the three-week treatment group (Fig. 6.7 L). Furthermore, PS-ASO treatment stimulated these apoptotic enzymes to a greater extent than DTX treatment alone.

Rationale for Targeting Mutated *H-Ras* and *c-raf-1* Genes Individually with ASOs

Following the successful inhibition of chemically induced hepatocellular carcinoma (HCC) in rats using an ASO against the mutated *H-Ras* gene, we sought to compare the anticancer effects of targeting the mutated *H-Ras* and *c-raf-1* genes individually with their respective ASOs. This approach aims to identify potential differences in therapeutic outcomes.

The Ras protein and its downstream effectors play critical roles in regulating cell survival, differentiation, proliferation, and apoptosis. Ras primarily mediates signaling through two key survival pathways: the Ras-Raf-MEK-ERK pathway and the Ras-PI3K-PKD-AKT pathway (Malumbres and Barbacid, 2003), alongside additional apoptotic and signaling cascades (Malumbres and Barbacid, 2003; Aoki et al. 2008). The downstream effector c-raf-1 kinase is a pivotal signaling molecule within the Ras-Raf-MEK-ERK pathway, transmitting signals related to differentiation, mitogenic activity, and carcinogenesis to MEK and ERK kinases (Bahar et al. 2023). Dysregulation of c-raf-1 disrupts normal cell division and development and is closely linked to oncogenesis (McCubrey et al. 2007).

Oncogenic Ras also influences the caspase-3 pathway, which exhibits both pro-apoptotic and pro-survival roles in cancer through deregulation of multiple signaling routes, including PI3K and MAPK pathways (Plati et al. 2011). Importantly, the mutant *c-raf-1* gene acts as an oncogene and is overexpressed in nearly all HCC cases, playing a crucial role in disease progression (Hwang et al. 2004).

Nanoscale Topography of ASOs

Atomic force microscopy (AFM) was employed to characterize the nanoscale topography of the ASOs. AFM provides high-resolution imaging of soft biological materials under near-natural conditions. The analysis revealed that the ASOs exhibited a well-dispersed distribution with nanoscale sizes, confirming their nanosize and distinct topographical features (Fig. 6.8 A-B).

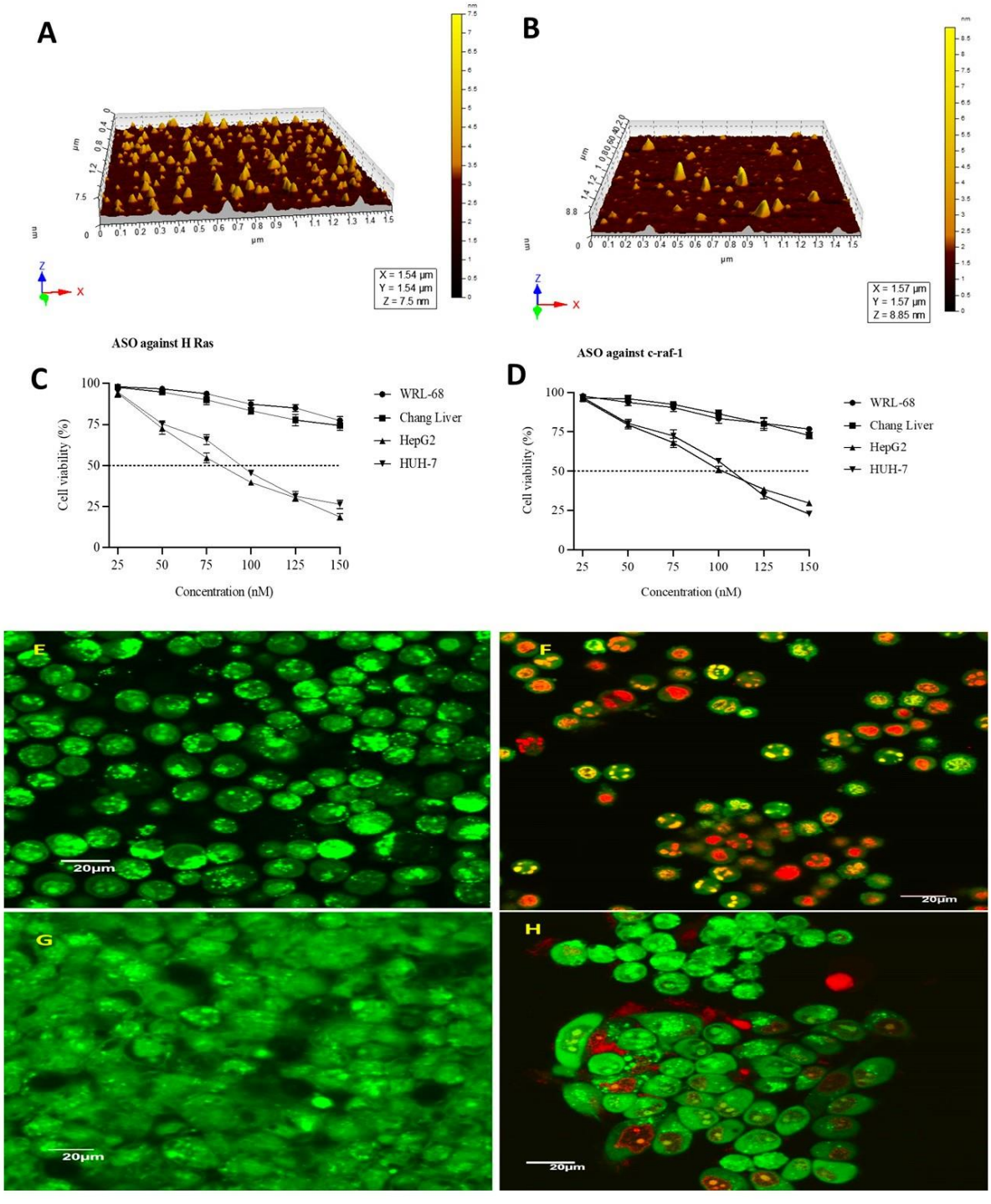

Fig. 6.8. Atomic force microscopy (AFM) data of fluorescein amidite (FAM)-labeled phophotothoate-backbone modified experimental antisense oligomers (ASOs), cell viability analysis of the experimental ASOs in two HCC cell lines, HepG2 and Huh7, and two normal

cell lines Chang liver and Wrl-68, and confocal microscopic photograph of cellular apoptosis of HCC cells received ASO treatments for 12 h and 48 h ASO treatments. Morphological topography analyzed by AFM of (A) ASO1 (B) ASO2; cell viability assay of various cell lines (C) upon ASO1 treatment (D) upon ASO2 treatment; Apoptosis analysis study by flow cytometer (E) HepG2 cells treated with ASO1 for 12 h shows early apoptosis in cells with bright green nucleus, perinuclear bright green small particle-like chromatin condensation, and nuclear fragmentation (F) HepG2 cells treated with ASO1 for 48 h, having more late apoptotic cells and the cells appeared yellow to orange to red nuclei with condensed or fragmented chromatin due to high amount of ethidium bromide uptake (G) Huh7 cells treated with ASO2 for 12 h shows early apoptotic cells, (H) Huh7 cells treated with ASO2 for 48 h, shows less late apoptotic cells compared to ASO1 treatment for 48 h.

MTT assay data show mean ± SD, n= 3. *$p<0.05$ was considered as the statistical level of significance when data of the treated Huh7 cells were compared to the treated HepG2 cells.

ASO against mutated H-ras and ASO against c-raf.1 showed variable but close IC50 in human HCC cells but not in normal human liver cells

The IC50 value in a cell viability assay shows the concentration of a substance that can prevent 50% of cells from surviving. We measured the ASOs' IC50 values in two human HCC cell lines (HpG2 and Huh7) as well as two normal hepatic cell lines (Wrl68 and Chang liver). ASO1 and ASO2 had respective IC50 values of 90.87±2.43 nM and 115.65±3.28 nM in HepG2 cells, and 102.21± 2.88 and 118.98± 3.42 nM in Huh7 cells (Fig. 6.8 C-D). Between the cell types, the IC50 results differed significantly ($p<0.05$) in HepG2 cells but not in Huh7 cells.

ASO treatment against mutated H-ras showed better induction ability of apoptosis and MMP in HepG2 and Huh7 cells than ASO treatment against c-raf.1

In multicellular organisms, the most frequent method of cellular death is apoptosis, or programmed cell death. When oxidative stress levels climb above a certain threshold, mitochondrial malfunction and apoptosis are triggered. Numerous morphological changes, including membrane blebbing, cell shrinkage, chromatin condensation, apoptotic body formation, and nuclear disintegration, are brought on by apoptosis (Chakraborty et al. 2020).

Acridine orange (AO) uptake causes the cells to fluoresce green, and early apoptotic cells are seen in a bright green nucleus with perinuclear brilliant green particulate chromatin condensation. Prominent ethidium bromide (EB) uptake caused late apoptotic cells to show up as orange to red nuclei with broken or contracted chromatin called chromatin condensation. Following ASO1/2 treatment for 12 and 48 hours, we qualitatively examined the early/late apoptosis in both cancer cell lines using confocal microscopy (Fig. 6.8 E-H) and quantitatively using annexin v-FITC/PI (Fig. 6.9 A) and MMP utilizing JC-1 (Fig. 6.9 B) by flow cytometry only for ASO1 in HepG2/Huh7 cells. This is because, in comparison to the previous research conducted here, ASO2 shown less HCC inhibitory potential than ASO1.

HepG2 cells treated with ASO1 for 12 hours (Fig. 6.8E) and treated with ASO1 for 48 hours (Fig. 6.8F), and Huh7 cells treated with ASO1 for 12 hours (Fig. 6.8G) and treated with ASO1 for 48 hours (Fig. 6.8H) are shown in confocal microscopic images. Bright green nucleus, nuclear fragmentation, and perinuclear bright green small particles that show chromatin condensation were all signs of early apoptosis in cells. HepG2 cells treated with ASO1 for 48 hours showed more late apoptotic cells, having yellow to orange to red nuclei with constricted or fragmented chromatin as a result of significant ethidium bromide uptake. Compared to ASO1 treatment for 48 hours, Huh7 cells treated with ASO2 for 48 hours displayed fewer late apoptotic cells.

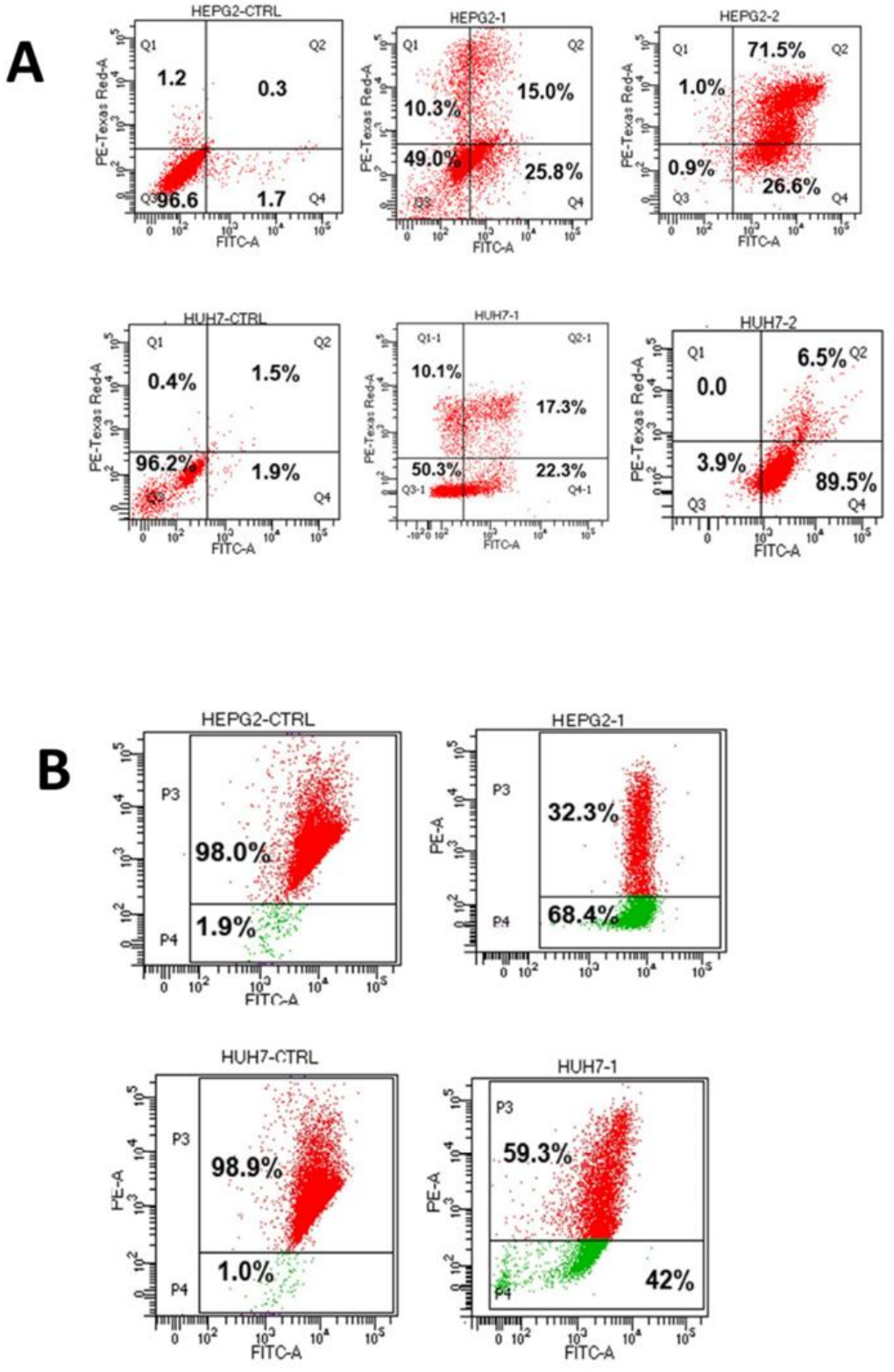

Fig. 6.9. Quantification of cellular apoptosis and mitochondrial membrane depolarization using flow cytometer on HepG2/Huh cells received experimental treatments. (**A)** In vitro

cellular apoptosis study through Annexin V-FITC/PI in HepG2 cells. The first row, first graph from the left, control HepG2 received no treatment; 2nd graph, HepG2 cells received ASO2 treatment against *c-raf.1* gene for 48 h; the third figure, HepG2 cells received ASO1 against mutated *H-ras* gene for 48 h. The second row, first graph from the left, control Huh7 cells received no treatment; 2nd graph, Huh7 cells received ASO2 (treatment against *c-raf.1* gene) for 48 h; the third figure, Huh7cells received ASO1 (against mutated *H-ras* gene) for 48 h. In each graph, anti-clockwise the first quadrant represents live cells, 2nd quadrant represents early apoptotic cells, 3rd quadrant represent late apoptotic cells, and the 4th quadrant represents death cells **(B)** In vitro cellular mitochondrial membrane depolarization estimation in HepG2/Huh7 cells. The first row, first graph from the left, control HepG2 received no treatment; 2nd graph, HepG2 cells received ASO1 treatment against *H-ras* gene for 48 h. The second row, first graph from the left, control Huh7 cells received no treatment; 2nd graph, Huh7 cells received ASO1 treatment for 48 h. P4 quadrant represents depolarized mitochondria (green) and P3 represents active mitochondria.

The quantitative analysis using a flow cytometer, ASO1 therapy against mutant H-Ras showed 98.1% overall apoptosis in HepG2 cells and 96% in Huh7 cells (Fig. 6.9 A). Although ASO2 produced 89.5% early apoptosis, but it showed only 6.5% late apoptosis in Huh7 cells. ASO1 caused 71.5% late apoptosis in HepG2 cells with 26.6% early apoptosis, suggesting that ASO1 is more effective in HepG2 cells. For MMP investigation with JC-1 staining, live cells containing normally active mitochondria show normal MMP values and the mitochondria produce JC-1 aggregates with red fluoresce. JC-1, on the other hand, is a green-fluorescent monomer seen in apoptotic cells with reduced MMP. The red-green fluorescence ratio changed more when H-Ras was treated than when c-raf.1 was treated (68.4% versus 42%) with the respective ASOs (Fig. 6.9 B). This suggests that ASO1 causes more severe mitochondrial depolarization in malignant hepatocytes. ASO1 (ASO against mutant H-Ras) was shown to be more effective than ASO against c-raf.1 (ASO2) in causing both cell types to undergo apoptosis and mitochondrial depolarization. There is always a correlation between increased mitochondrial depolarization and increased apoptosis (Chakraborty et al. 2020).

ASOs were distributed well in normal rat liver

High-resolution images of deep-seated cells and tissues stained with fluorescent probes are the main output of confocal laser scanning microscopy, which blocks out-of-focus light and creates a point source of light. It was found that 12 hours after being administered intravenously, FAM-labeled ASOs were well-internalized in the liver of normal rats (Fig. 6.10 A-B) (Monia et al. 1999; Mukherjee et al. 2024).

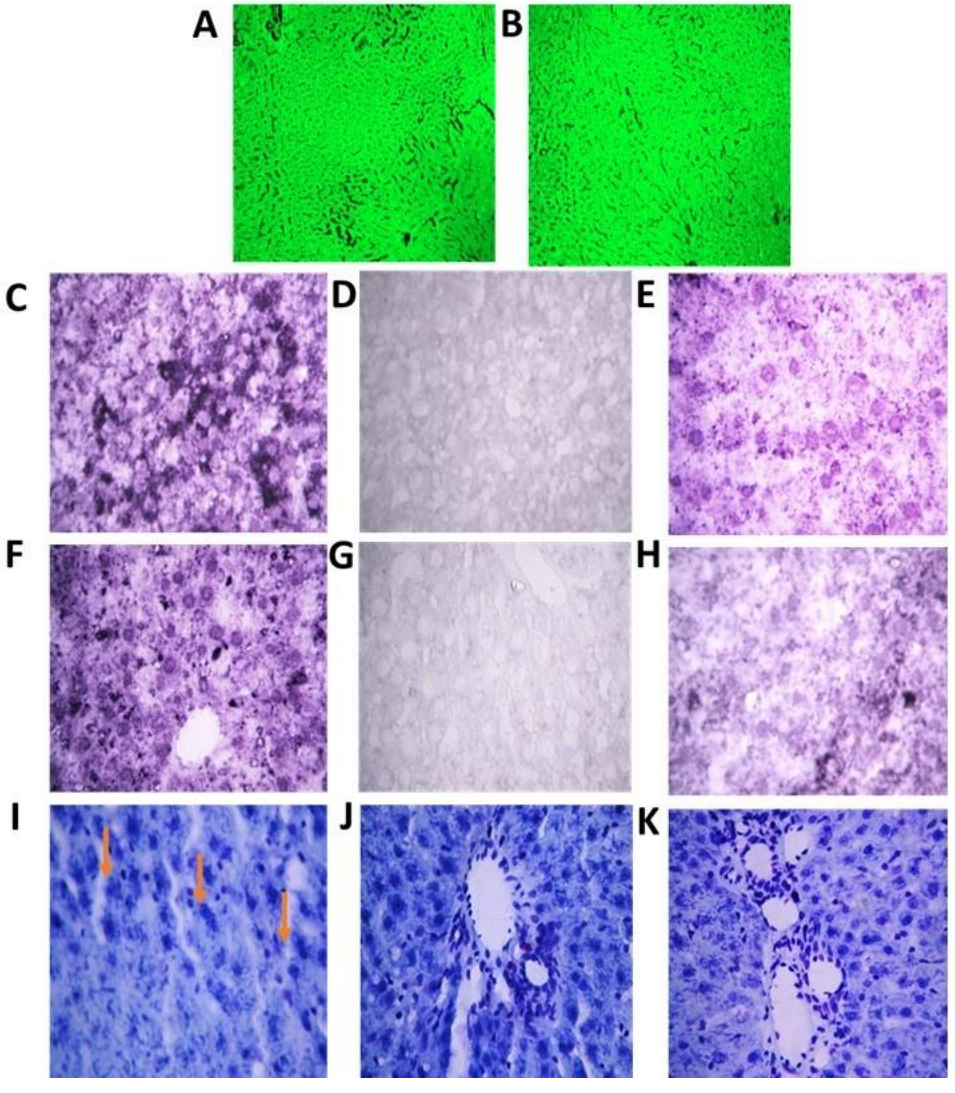

Fig. 6.10. Hepatic ASO uptake, mutated *H-ras*/ *c-raf.1* gene expressions (studied by in situ hybridization), histological changes in various experimental groups of rats. **A**. confocal microscopic photograph of liver section, 6 h after FAM-ASO1 administration (magnification 10X); **B.** confocal microscopic photograph of liver section, 6 h after FAM-ASO2

administration (magnification 10X); **C.** hepatic section of carcinogen-induced HCC rats (carcinogen control), showing mutated *H-ras* gene expression; **D.** corresponding sense control; **E.** carcinogen-treated rats treated with ASO1; **F.** hepatic section of carcinogen-induced HCC rats (carcinogen control), showing *c-raf.1* gene expression; **G.** corresponding sense control; **H.** carcinogen-treated rats treated with ASO2; Representative photographs of liver sections of **I.** carcinogen-induced HCC rats (carcinogen control) upon toluidine blue staining, showing tumor area by yellow arrow heads; **J.** carcinogen-treated rats treated with ASO1, showing predominant hepatic improvement; **K.** carcinogen-treated rats treated with ASO2, showing hepatic improvement. Magnification of the photographs 40X, if not otherwise mentioned.

Respective ASO treatment reduced mutated H-Ras/c-raf.1 gene expression and remarkably altered hepatic altered focal lesion count and inhibited tumor incidences

Mutations in the *raf.1* gene and the *H-Ras* gene generally encourage the development of several cancer forms, including HCC. The cancer-control HCC rat liver was discovered to have a high level of overexpression of mutant H-ras and c-raf.1, which were significantly downregulated after receiving the corresponding ASO treatment (Fig. 6.10 C-H). Histomorphologically distinct from a normal liver, hepatic altered focal lesions, or foci (HAF), comprise both preneoplastic and neoplastic liver lesions that can produce aberrant solid or liquid masses, resulting in neoplasia. First, when rats were given carcinogens, no tumor development was seen after receiving ASO therapy. Multiple liver tumors were simultaneously observed in carcinogen control rats (Fig. 6.10 I-K). In rats given carcinogens and treated with ASOs, ASO1 medication decreased the number and size of hepatic lesions more than ASO2 treatment (Table 6.4).

Table 6.4 Tumor incidences, and number and size of hepatic altered foci (HAF) in experimental rats

Animal groups	**Tumor incidences (out of six animals in each group)**	**% Hepatic altered foci (HAF)/cm^2 hepatic tissue area**		
		< 1 mm	**1 mm-3 mm**	**>3mm**
Group A (normal control)	0/5			
Group B (carcinogen control)	5/5	17.5±1.2	34.2±2.4*	48.3±3.2*
Group C (carcinogen-treated rats treated with ASO against mutated H-ras mRNA for 6 weeks)	0/5	84.2±2.9$^{\#}$	14±1.8*,$^{\#}$	1.8±1.2*,$^{\#}$
Group D (carcinogen-treated rats treated with ASO against c-raf.1 mRNA for 6 weeks)	0/5	52.1±3.2$^{\#}$	34.6±2.6*,$^{\#}$	13.3±2.1*,$^{\#}$

Data show mean±SD (n = 5); Data were analyzed by one way ANOVA, followed by Dunnett's test.

*$p < 0.05$, HAF incidences when compared to the size (< 1 mm) of the same group

$^{\#}p < 0.05$, HAF incidences when compared to the percentage count of sizes against Group B rats

ASO treatment enhances p53 expression while reducing Hep Par 1 and HSP70 levels in rat HCC

Overexpression of the tumor suppressor protein p53 is associated with both well-differentiated and poorly differentiated hepatocellular carcinoma (HCC), playing a key role in regulating gene expression during the cell cycle. Hep Par 1 serves as a highly specific marker for HCC, whereas HSP70 promotes tumor growth by supporting the proliferation of cancer cells. Immunohistochemical analysis was used to assess the expression of these three critical proteins—p53, Hep Par 1, and HSP70. Rats exposed to carcinogens exhibited a significant increase in p53 expression following ASO treatment, with ASO1 producing a notably greater upregulation ($p < 0.05$) compared to ASO2 (Table 6.5). Conversely, carcinogen-treated rats receiving ASO therapy showed a marked reduction in Hep Par 1 and HSP70 expression (Fig. 6.11), with ASO1 achieving the most pronounced decrease in both proteins.

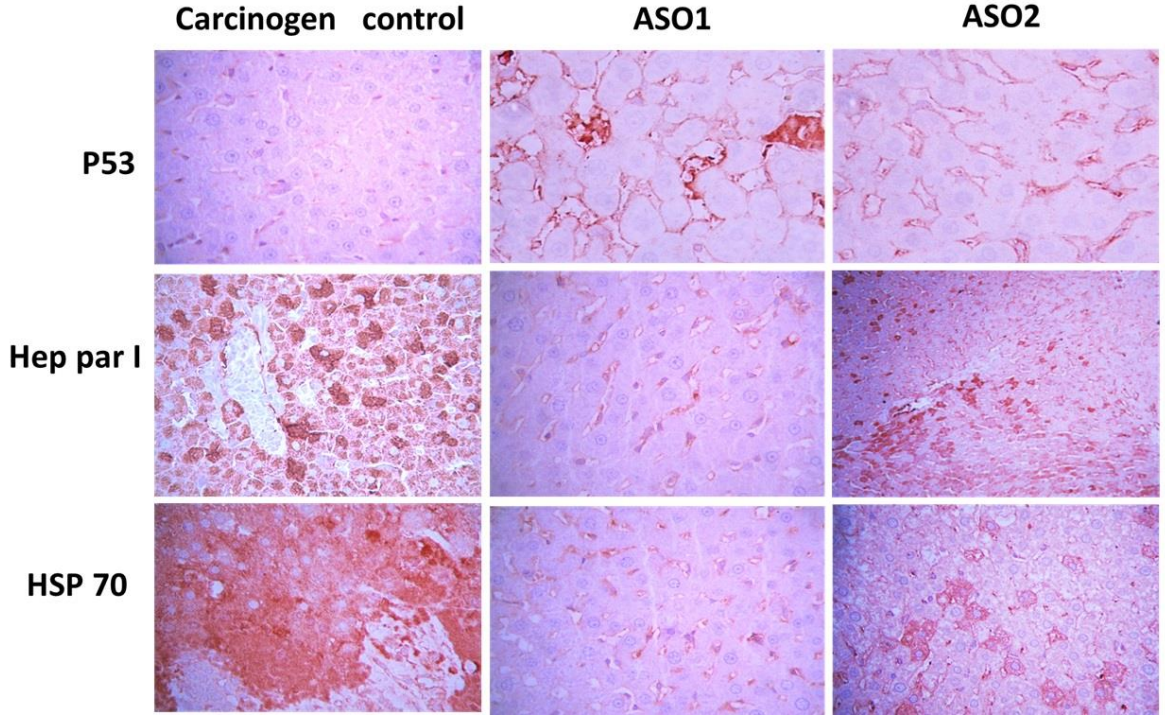

Fig. 6.11. Expression of p53, Hep par I, and HSP70 proteins (by immunohistochemical method) in carcinogen-induced HCC rats (carcinogen control), and carcinogen-induced HCC rats treated with ASO1 and ASO2. B. Magnification of the photographs 40X.

Table 6.5 Combined semi-quantitative scoring (CSS) analysis of data of mutated H-Ras/*c-raf.1* gene expressions by in-situ hybridization, and p53, Hep par-I, and HSP70 protein expressions by immunohistochemistry of experimental rats

Treatment received/ Expression type	Mutated *H-Ras* gene expression [a](CSS)	*c-raf.1* gene expression (CSS)	p53 protein expression (CSS)	Hep par-I protein expression (CSS)	HSP70 protein expression (CSS)
Carcinogen control rats	73.6 ±3.2	58.4 ±2.5	2.4 ±0.16	68.3 ±2.8	64.3 ±4.6
Carcinogen-treated rats received ASO1 treatment for 6-weeks	7.8 ±0.3*,#		26.5 ±2.2*,#	8.4 ±0.7*,#	13.6 ±3.5*,#
Carcinogen-treated rats received ASO2 treatment for 6-weeks		9.6 ±1.8*	18.7 ±1.6*	20.6 ±2.1*	36.5 ±3.6*

[a]CSS was done based on staining intensity and % of stained cells/cm^2 area. Data show mean ± standard deviation and were analyzed based on 20 slides (20x3 observations) in each case.

*$p<0.05$, when data were compared with respect to carcinogen control, assessed by one-way ANOVA followed by Dunnett's test.

#$p<0.05$, when compared the data of carcinogen-treated rats received ASO1 (against mutated H-ras) against data of carcinogen-treated rats received ASO2 (against c-raf.1), assessed by one-way ANOVA followed by Student's t-test.

ASO treatments variably induced caspase-3 and -9 protein levels in HCC rats

A favorable sign of apoptosis and the effectiveness of cancer treatment is the overexpression of caspase 3. The initiator caspase is broken down by caspase-9, which then triggers effector caspases, such as caspase-3. Both ASO treatments triggered the main intrinsic apoptosis markers, caspase-3 and caspase-9, confirming their function in inducing apoptosis in cancer cells (Fig. 6.12 A). ASO1 showed better performances in inducing both enzyme activities.

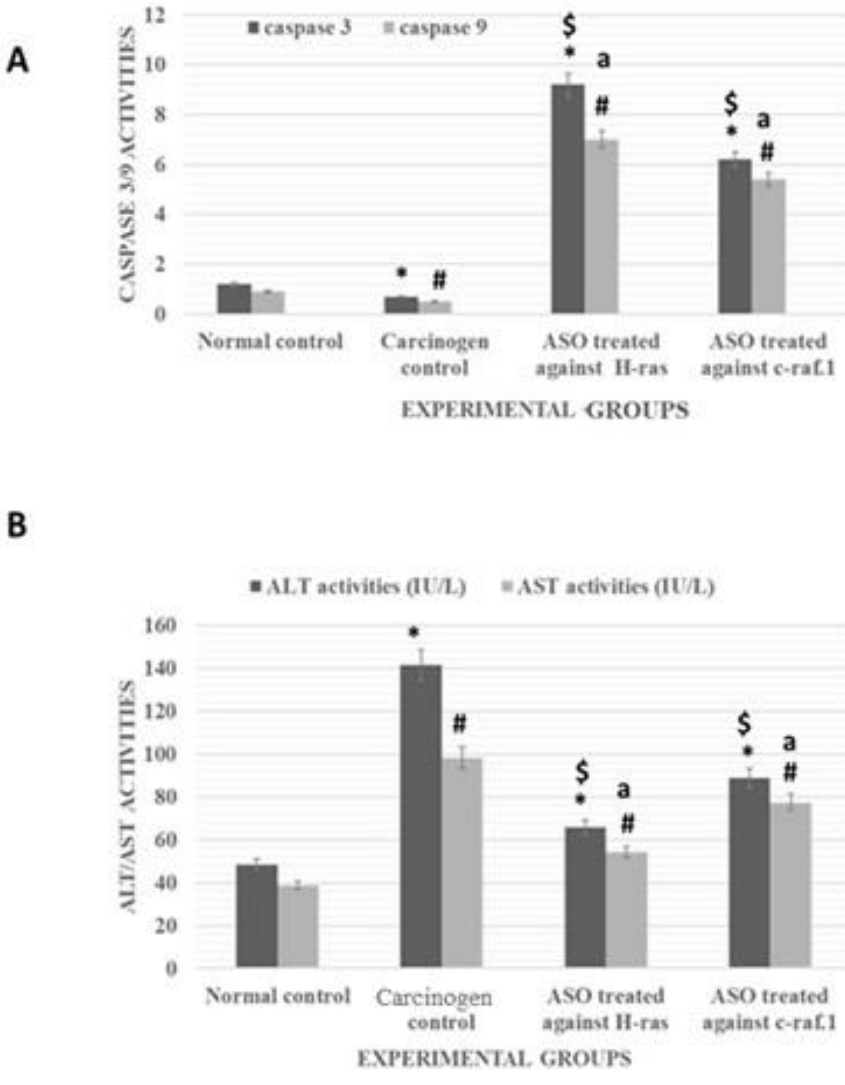

Fig. 6.12. Hepatic caspase 3/9 activities and blood levels of hepatic marker enzymes in experimental rats.

A. Caspase 3 and caspase 9 activities were measured in liver homogenates from the experimental rats and are presented as fold changes relative to the carcinogen control group. **B.** Blood levels of ALT and AST were assessed in various control and treated groups. Data are expressed as mean ± standard deviation (n = 3). Statistical significance is indicated as follows: $p < 0.05$ and #p < 0.05 denote comparisons against the respective normal control rats, while $p < 0.05$ and ap < 0.05 indicate significance compared to the carcinogen control HCC rats. One-way ANOVA followed by Dunnett's t-test was used for analysis.

ASO treatment normalizes hepatic marker enzymes in carcinogen-exposed rats
Aspartate aminotransferase (AST), a microsomal enzyme, and alanine aminotransferase (ALT), a hepatocellular cytosolic enzyme, are well-established markers of liver injury. In carcinogen-

exposed rats, both ASO treatments significantly reduced these enzyme levels toward normal ranges (Fig. 6.12 B). Notably, ASO1 treatment resulted in a greater decrease in enzyme levels compared to ASO2.

These findings highlight the potential of PS-ASO gene therapy targeting the mutated *H-Ras* gene as a promising approach for hepatocellular carcinoma treatment. Further investigations are warranted to explore its applicability and synergy with other cancer therapies.

Chapter 7

Discussion

Discussion

Analyses of the mass, surface charge, and three-dimensional structure, and nucleotide chain length were first determined. The height, zeta potential, and molecular mass were all increased by FAM-conjugated PS-ASO. The length of PS-ASOs, as determined by agarose gel electrophoresis, was 21 nucleotides. There were no odd changes in the characteristics of the FAM-conjugated PS-ASO. The spiral poly-crystalline structure of PS-ASOs was suggested by TEM and SAEDP experiments. It has been demonstrated that PS-oligomers have negatively charged backbones that bind to plasma proteins strongly in both humans and animals (rats), which results in their long-lasting effects and stability in blood (Shi et al. 2022; Radi 2019; Shen and Corey 2018). ASOs with phosphorothioate backbone modifications have been shown to have strong nuclease and metabolic stability, making them stable in serum (Kazemi et al. 2020; Miller and Harris 2016). Less than 3% hemolytic activity was observed between 5 and 100 nM of PS-ASO administered. PS-ASO can be safely administered intravenously because biomaterials are safe to use up to 5% hemolytic critical value (ISO/TR (International Organization for Standardization/Technical Report) 7406.46).

In liver uptake investigation, PS-ASOs were quickly dispersed throughout the liver and gradually gathered in the hepatocytes throughout the course of the study, for PS-ASOs do not interact with cytochrome and UDP-glucuronosyltransferase enzymes (Kazemi et al. 2020) and have a high nuclease stability (Miller and Harris 2016), they are metabolically stable in liver and blood.

The backbone chemistry of PS-ASOs primarily governs their pharmacokinetic profile, biodistribution, and plasma protein binding. PS-ASOs remained stable in both human and rat blood (Radi 2019; Cunningham et al. 2001). A significant decrease in the blood level of DTX was observed following a dose of Taxotere® compared to PS-ASO adminstration. Given its relatively longer blood residence time, PS-ASO demonstrated a longer duration of therapeutic activity. PS-ASO significantly increased tissue distribution and half-life may be due to increasing plasma protein binding and plasma stability as reported (Geary et al. 2015). The investigation confirms previous findings that PS-ASOs had a longer half-life in blood, lower overall clearance values, and a higher bioavailability (AUC) in experimental animals when compared to DTX. Phosphorothioate oligonucleotide binding to plasma proteins prolongs

inhibitory efficacy by improving tissue distribution and limiting glomerular filtration, which regulates PS-ASO excretion in the urine (Geary et al. 2015).

High quantities of PS-ASO were primarily found in the liver and kidney, the clearance organs, It was followed by the lungs and spleen. In spleen, concentration of PS-ASO was notably low. In the lungs, the PS-ASO concentration rose quickly from 6 to 8 hours. Enhanced PS-ASO levels in the liver and lungs were found, which could have shown the potential to reduce the risk of primary hepatic neoplasia, and metastatic lung tumors in the present investigation. Over time, the spleen also distributed an increasing amount of PS-ASO, although at a relatively modest concentration. Additionally, our confocal microscopic data corroborated the study.

Hepatocytes quickly import small, stable PS-ASOs through the use of endosomal membrane-bound scavenging receptor stabilin and clathrin-mediated endocytosis (Miller et al. 2016). Early endosomal pathway proteins Rab5C and EEA1 (early endosomal antigen 1, found in endosomal peripheral membrane with zinc dependent binding motifs) were well-known for phosphorothioate-ASO trafficking to endosomes in a variety of cancer cell lines, including HepG2 cells with liver carcinoma (Wang et al. 2016; Miller et al. 2018). Additionally, Rab7A and lysobisphosphatidic acid-rich late endosomes are crucial for PS-ASO escape during late endocytosis (Wang et al. 2017). PS-ASOs enter the cytoplasm by lysosomal escape (Wang et al. 2016; Miller et al. 2018), bind to the targeted site of overexpressed mRNA complementary sequences, for function (Bennett 2019). It results in the breakdown of mRNA by RNase and halts the production of the gene's protein (Crooke et al. 2018). In order to halt gene expression, PS-ASOs can also penetrate the nuclear membrane and enter the nucleus by blocking complementary sequences (Bennett 2019).

A carcinogenic insult results in a number of genetic and epigenetic modifications (Mukherjee et al. 2005), which induce significant biochemical and morphological changes in the cells, eventually leading to generate preneoplastic lesions (Mukherjee et al. 2022; 2005), The current experimental model showed several forms of preneoplastic lesions. Numerous preneoplastic lesions eventually progress to dysplastic nodules and neoplastic lesions (Mukherjee et al. 2022; 2005), The dysplastic/hyperplastic nodules ultimately give birth to HCC (Mukherjee et al. 2022; 2023). In comparison to the other treatments, six week-long PS-ASO treatment significantly slowed the development of HCC in the current experimental rat model by

reducing the number of larger lesions (>3mm-1mm) while retaining the highest percentage of small preneoplastic lesions (<1mm). The observation that tumor incidences did not occur in rats given carcinogen and received PS-ASO for six weeks further corroborated the findings. Metastatic lung cancers were seen in certain carcinogenic animals. PS-ASO/DTX therapy prevented the growth of these lung metastases.

Normal animals that got PS-ASO showed no significant histological alterations in their liver, kidney, lung, or spleen. High PS-ASO concentrations in healthy kidneys did not exhibit any histological signs of toxicity in the renal tissue. This conclusion was corroborated by a previous study that found few instances of non-adverse reversible kidney changes but no signs of kidney functional abnormalities in rats given PS-ASO over an extended period of time (Radi 2019). The kidneys, lungs, and liver, however, displayed some histological changes following a 6-week course of Taxotere® treatment, indicating DTX-mediated toxic manifestation in those tissues.

HCC tumor genesis, development, and maturation have all been closely linked to elevated *H-Ras* gene expression (Sun et al. 2022, Gedaly et al. 2010). Rats treated with the carcinogen and received PS-ASO long-term treatment showed the greatest inhibition of gene expression. This suggests that PS-ASO treatment for six weeks effectively suppressed *H-Ras* gne expression to prevent the progression of HCC. DTX may have functioned without interfering with the *Ras* gene because it did not primarily suppress *H-Ras* expression. To suppress HCC, DTX inhibits mitotic cell division (Shaw et al. 2017).

Hep par-1 is one most specific and sensitive immunohistochemical HCC marker for HCC, which detects hepatocyte differentiation throughout carcinogenesis. It is a monoclonal antibody (mAb). The mAb acts against carbonyl phosphate synthetase, an important enzyme of urea cycle, that occurs in mitochondria (Roncalli et al. 2011). Even for weakly differentiated HCC, Hep par-1 is a commonly used immunohistochemistry marker (Hanif and. Mansoor 2014). The observed results in the current investigation supported the very robust expression of protein Hep par-1 in carcinogen control rats (Hanif and. Mansoor 2014). The PS-ASO/Taxotere® therapies showed inhibitory effect on Hep par-1 protein production. PS-ASO long-term (6-week) treatment showed maximal protein reduction, indicating that the therapy showed the greatest efficacy in delaying the progression of HCC in rats.

Around 30% of HCC were positive CK-7 [27]. The malignancy of the bile ducts that join the liver and gallbladder, known as cholangiocarcinoma, also exhibits high expression of CK-7 (Rullier et al. 2000). The greatest reduction in CK-7 positive cells and, hence, the greatest effectiveness in regulating CK-7 expression during the formation of HCC were observed with PS-ASO long-term treatment.

Because it develops intrahepatic metastases, HCC is different from other solid tumors and typically has a bad prognosis (Yang et al. 2017). Numerous investigations revealed a positive association between CD15 expression, intrahepatic metastases, and HCC (Szlasa et al. 2022; Torii et al. 1993). Intrahepatic metastasis was observed by the formation of numerous tumors in carcinogen-control animals and strong CD15 expression. The effectiveness of PS-ASO (for three weeks)/Taxotere® experimental treatments in reducing hepatic CD15 expression in rats treated with carcinogens further supports the treatment's ability to suppress intrahepatic metastases and the progression of HCC. On the other hand, sustained PS-ASO therapy showed an exceptionally high inhibitory efficacy of HCC tumor growth.

Apoptosis, DNA repair, senescence, cell cycle, cell death, and cellular metabolism are all regulated by the p53, a tumor suppressor gene, which is most commonly altered in liver cancer (Link and Iwakuma 2017). The carcinogen control rats showed very low p53 expression. Raised gankyrin, which regulates bis 26S proteasome, murine double minute 2, and p53-specific ubiquitin ligase all lower p53 levels in HCC (Iakova et al 2011; Ranjan et al. 2016). The dysregulation of miRNA-24 in HCC facilitates neoplastic invasion and metastasis by inhibiting p53 levels (Chen et al. 2016). The origin of HCC is aided by this decreased p53 activity. However, in rats given carcinogens, the experimental treatments exhibited increased p53 expression in the following order: PS-ASO treatment for 6 weeks> PS-ASO treatment for 3 weeks> Taxotere® treatment. This suggests that the experimental therapies may play a role in inhibiting the HCC development by raising p53 levels.

The ultrastructure of the normal (control) liver showed a packed array of tiny, spherical hepatocytes. Short microvilli on the cell surface, portal and artery canals, and sinusoidal gaps are all clearly visible (Russo et al. 1977; Schulze et al. 2019). In their ultrastructure, hepatocytes have a rough surface due to the presence of an apical (bile canaliculated) and a basolateral

(sinusoidal) membrane domain (Schulze et al. 2019). Hepatocytes were polygonal in three-dimensional look and made contact with neighboring hepatocytes or sinusoids. Bile canaliculi were created by modifying a part of the hepatocytic lateral surface (Schulze et al. 2019). A small number of microvilli faced into bile canaliculi, while the majority faced sinusoidal gaps. Hepatocytes in livers with carcinogen control (HCC) were primarily densely packed, and microvilli had a distinctly growing aspect. Numerous microvilli seen on the surface of hepatocytes are known to aid in the exchange of waste products and nutrients through the plasma (Ren 1991). High tumor growth and a high potential for metastasis are directly linked to increased microvilli (Ren 1991). Actually, three out of five rats in the carcinogen control group had lung metastases, and significant tumor development was seen in these animals. However, after receiving PS-ASO, the neoplastic hepatocytes seemed to have significantly improved in comparison to the normal hepatocytes in the carcinogen control rats. According to data, PS-ASO therapy unquestionably brought the malignant hepatocytes' ultrastructure closer to normal.

The ALK, SGPT, and SGOT blood levels showed liver health or improvements in liver health as a result of the experimental therapy. The findings from long-term PS-ASO treatment indicated that maximum improvement of liver health was achieved by the therapy. Furthermore, activated caspase-9 changes the shape of the mitochondria and the formation of reactive oxygen species by converting Bid into tBid. Caspases 3 then prevent the production of ROS, which effectively triggers apoptosis (Brentnall et al. 2013; Sen et al. 2021). Prolonged PS-ASO therapy significantly increased caspase 3 and caspase 9 activities, suggesting that PS-ASO regulated the pathophysiology of HCC by encouraging apoptosis.

Following the effective ASO (against the mutated *H-Ras* gene)-induced inhibition of chemically induced HCC in rats, we aimed to compare the HCC inhibitory effect of respective ASO-mediated intervention of the mutated *H-Ras/c-raf.1* gene separately and investigate potential differences in treatment outcomes. The justification for blocking each of these two genes separately is explained in the following paragraph.

The Ras protein and its downstream effectors are necessary for the proliferation, differentiation, death, and survival of cells. In addition to an apoptotic route and other pathways (Malumbres

and Barbacid, 2003; Aoki et al. 2008), Ras primarily mediates the signaling in two important survival pathways: Ras-Raf-MEK-ERK pathway and Ras-PI3K-PKD-AKT pathway (Malumbres and Barbacid, 2003). A crucial signaling molecule in the Ras-Raf-MEK-ERK pathway, c-raf-1 kinase relays information about differentiation, mitogenic activity, and carcinogenesis to the downstream kinases MEK and ERK (Bahar et al. 2023). It is essential for regulating cell division and development, and dysregulation of it is linked to oncogenesis (McCubrey et al. 2007). Furthermore, by deregulating numerous pathways, including PI3K and MAPK, oncogenic Ras has a complicated influence on the caspase-3 pathway, which has pro-apoptotic and pro-survival effects in cancer (Plati et al. 2011). According to Hwang et al. (2004), the mutant *c-raf-1* gene is an oncogene that is overexpressed in almost all cases of HCC and is crucial to the progression of the illness. Hence, we intended to explore blocking which gene (mutated *H-Ras/ c-raf.1*) is more potential to inhibit HCC.

In order to suppress the expression of the *Ras* or *Raf* genes in rats that had specific mutations during the development of hepatocellular carcinoma, we employed the corresponding antisense oligomers as previously indicated. Using the MTT assay, the IC50 values were first calculated in two hepatocellular carcinoma cell lines, HepG2 and Huh7. In both cell types, HepG2 and Huh7, ASO1 and ASO2 treatments exhibited variable IC50 values, but they followed a similar pattern. ASO1 showed lower IC50 values, indicating potentially more toxic to the cancer cells. The Chang liver and Wrl68, two normal liver cell lines, did not exhibit any discernible toxic effects from ASOs. The IC50 dose of the corresponding ASO was administered to the hepatocellular carcinoma cell lines, HepG2 and Huh7, for further investigation of apoptosis and mitochondrial depolarization as mitochondrial depolarization is always associated with apoptosis (Chakraborty et al. 2020). ASO1 treatment showed increased apoptosis and in mitochondrial depolarization in HepG2 cells than Huh7 cells, indicating blocking H-Ras has greater effect in HepG2 cells.

ASO treatment considerably ($p<0.05$) decreased HAF counts in number and size of the lesions, as seen in our histological data analysis. Crucially, no tumor incidence was noted while experimental rats received both carcinogen and ASO medication, offering potential support of ability of ASOs to prevent the growth of HCC. ASO1 showed lower large (>3 mm) HAF counts than ASO2 did, indicating that ASO1 was more effective in delaying HCC development.

The overexpressed mutated *Ras/c-raf.1* gene may have been a significant factor in the formation of cancer because ASO therapy mostly decreased the expression of the mutated *Ras/c-raf.1* in carcinogen-treated rats, while normal rats showed no expression of the mutated genes (Hwang et al. 2004). Comparing two ASOs, however, revealed that ASO1 had significantly lower gene expression than ASO2.

Under normal, healthy circumstances, the tumor suppressor gene *p53* repairs DNA, and causes apoptosis induction, and cell cycle regulation in order to advance to the S-phase of the cell cycle (Nayak et al. 2011). However, a number of malignancies, including HCC, have very little to minimal expression (Nayak et al. 2011). ASO1 treatment exhibited higher levels of p53 protein expression than ASO2, and ASO treatments increased p53 protein expression in carcinogen-treated rats, suggesting their potential for p53-mediated HCC suppression. Therapeutic effects on HCC, such as inducing apoptosis and inhibiting cell proliferation, are shown by high p53 expression in vivo (Guan et al. 2007). An immunohistochemistry gold flag for HCC is the overexpression of Hep par-1 in the liver (Hanif et al. 2014). In the urea cycle, this monoclonal antibody, which is found in mitochondria, works against carbonyl phosphate synthetase. In the development of HCC, it is an accurate indicator of hepatocellular differentiation (Roncalli et al. 2011). There have been numerous reports of elevated HSP70 gene and protein expressions in patients with early-stage HCC and HCC (Mohamed and Tealeb 2022). The HSP70 protein promotes the cancer survival autophagy process, stabilizes lysosomes, and commonly builds up in the lysosomal membrane of cancerous cells (Murphy 2013). Furthermore, HSP 70 suppresses apoptosis, inhibits the formation of apoptosome complexes, and slows caspase recruitment after binding with Apaf-1 and without being linked to procaspase-9 (Beere et al. 2000), all of which contribute to the advancement of HCC. ASO therapy demonstrated a significant decrease in HSP 70 and Hep par-I. When compared to ASO2, ASO1 shown a significant decrease in both proteins. The results imply that the Hep par-1 and HSP70 proteins were suppressed by both ASOs. The HCC inhibitory potential was more prominent after ASO1 therapy.

It is known that HCC overexpresses Caspase-3, a downstream effector cysteine protease of the apoptosis pathway (Persad et al. 2004). Apoptosis is the result of effector caspases, including

caspase 3, which are activated by the cleavage of the initiator caspase-9 (Persad et al. 2004; Hu et al. 2014). The current investigation found that both ASOs' apoptotic capability in hepatocarcinogenesis was further supported by the primarily elevated levels of caspase-3 and 9 following treatment. Compared to ASO2, ASO that suppresses the expression of the mutant *H-Ras* gene demonstrated greater promise.

Cirrhosis, HCC, and aberrant liver function have all been linked to elevated ALT or AST values as independent prognostic markers (Hernaez et al. 2013). Both ASOs significantly decreased the levels of the enzymes, but ASO1 performed better in improving HCC with little to no significant harm. This suggests that ASOs can improve hepatocarcinogenesis.

Therefore, both ASOs may cause apoptosis while inhibiting HCC. But the ASO was more effective at blocking the mutant *H-Ras*. Our findings indicate that ASO treatment may affect several additional signaling pathways (Malumbres and Barbacid 2003; Aoki et al. 2008), as seen by changes in the levels of the proteins under investigation, including p53, Hep par-1, and HSP70, and may promote the growth and progression of HCC.

Apoptosis, differentiation, survival, and cell proliferation all depend on the Ras protein and its downstream effectors. Together with an apoptotic route and other pathways (Malumbres and Barbacid 2003; Aoki et al. 2008; Plati et al. 2011), Ras mainly mediates the signaling of two important survival pathways: Ras-Raf-MEK-ERK pathway and Ras-PI3K-PKD-AKT pathway (Malumbres and Barbacid 2003). By dysregulating several pathways, including PI3K and MAPK, oncogenic Ras has a complicated effect on the caspase-3 pathway, which has pro-apoptotic and pro-survival effects in cancer (Plati et al. 2011). According to the study, Ras signaling suppression impacts the mitochondrial pathway, which in turn causes caspase-9 activation, leading to apoptotic cell death (Bahar et al. 2023).

Inhibiting the mutant *H-Ras* gene with ASO1 in this work resulted in a significant rise in mitochondrial depolarization and increased caspase 3/ caspase 9 activities, which in turn prevented the growth of cancer (Fig. 7). A crucial signaling protein in the Ras-Raf-MEK-ERK pathway, c-raf-1 kinase is in charge of communicating with the downstream kinases MEK and ERK about differentiation, mitogenic activity, and carcinogenesis (Bahar et al. 2023). It is

crucial for controlling cell division and development, and cancer start is associated with its dysregulation (McCubrey et al. 2007). It is known that the mutant *c-raf-1* gene is an oncogene that is overexpressed in nearly all HCC cases (Hwang et al. 2004) and is essential to the disease's development. It interacts with proteins like Bcl-2 and Bad (McCubrey et al. 2007) and activates caspase-3 (Bahar et al. 2023) to enhance apoptosis, according to reports that it plays a role in the process. According to our research, ASO2's suppression of mutant c-raf-1 caused HCC cells to undergo apoptosis and activated caspase-3 and caspase-9, which together prevented tumor growth and reduced the progression of HCC in the experimental rats. While H-Ras and c-raf.1 are part of the same Ras-Raf-MEK-ERK pathway, both ASO1/2 gene inhibitions variably suppressed HCC by modulating other HCC oncogenic proteins such as HSP70, p53, and Hep par 1 to varying degrees.

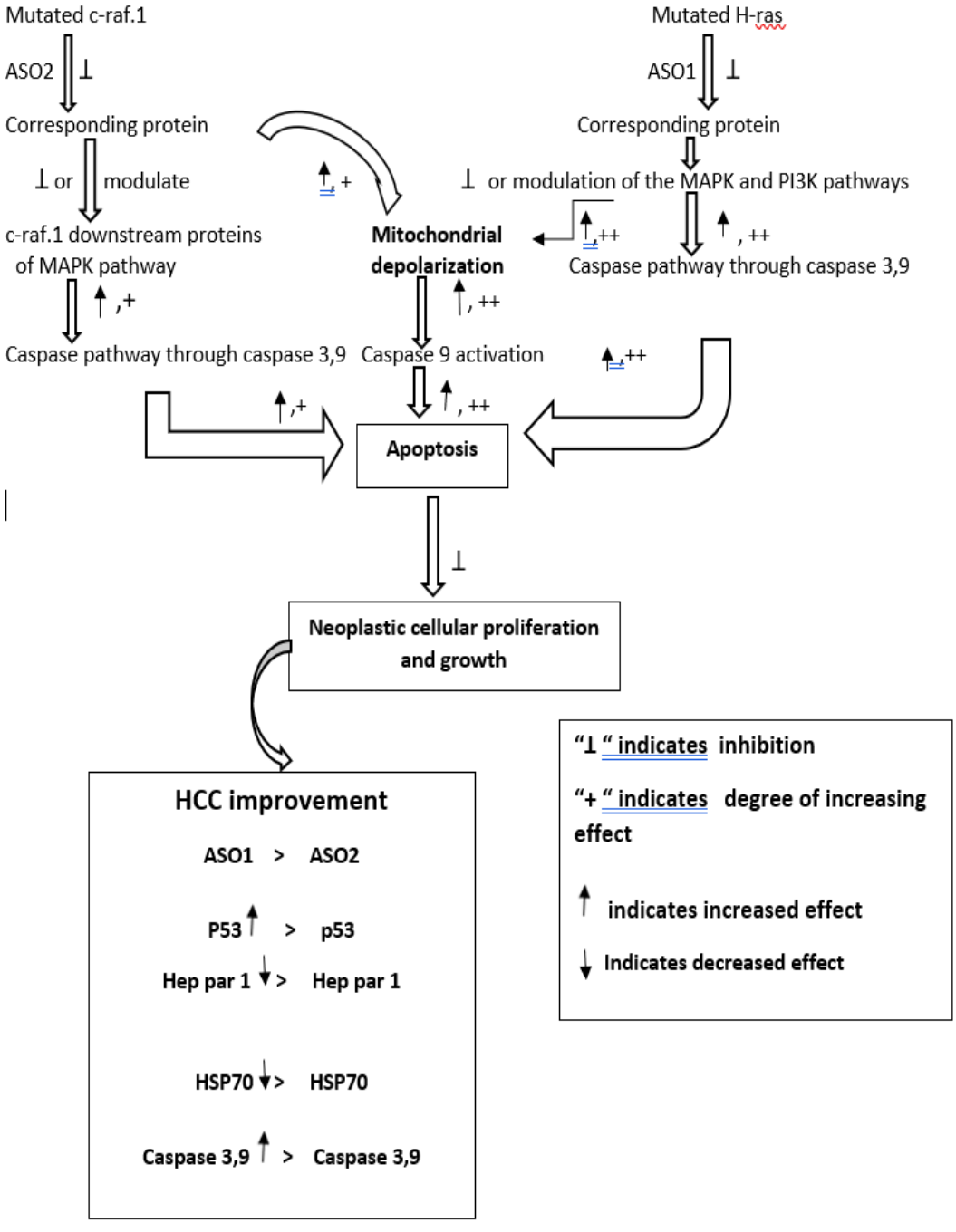

Fig.7. A mechanistic insight of ASO1/ ASO2-mediated inhibition of mutated *H-Ras/ c-raf.1* gene in inhibiting chemically-induced HCC in rats with a comparative approach

By increasing the caspase 3/caspase 9 activities through the manipulation of many Ras-related pathways, restricting Ras rather than c-raf.1 may therefore have a significant impact on the suppression of HCC. This implies that a key role for the *H-Ras* gene may be found in the signal transduction pathways that underlie molecular oncogenesis.

In particular, ASO's capacity to inhibit the mutant *Ras* gene was more potent than that *of c-raf.1* gene inhibition in suppressing HCC. Therefore, as this study investigated, ASO targeting the mutant *H-Ras* gene may have a more comprehensive impact in suppressing numerous proteins connected to the development of HCC.

Thus, by increasing caspase 3/caspase 9 activities through the manipulation of many Ras-related pathways, concentrating on the inhibition of Ras rather than c-raf.1 could significantly impact the suppression of HCC. Accordingly, the *H-Ras* gene may play a significant role in the signaling pathways that underlie molecular oncogenesis

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Particularly, ASO-induced suppression of the mutant *Ras* gene shown a stronger potential to reduce HCC than *c-raf.1* gene inhibition. As this study explores, targeting the mutant *H-Ras* gene with ASO may have a more widespread impact in suppressing numerous proteins connected to the development of HCC.

Chapter 8

Summary & Conclusion

Summary and conclusion

Hepatocellular carcinoma (HCC), a disease with a high fatality rate and a major contributor to cancer-related deaths globally, is typically detected at an advanced stage, when chemotherapeutic intervention is the only available treatment. Most anti-cancer medications are extremely cytotoxic. Most chemotherapeutic alternatives fall well short of expectations. On the other hand, compared to other chemotherapeutics, genetic therapies, such as antisense oligomer (ASO), demonstrated noteworthy HCC inhibitory capability without significant toxicity. Here, we first evaluated the effectiveness of ASO treatment against mutant *H-Ras* gene in suppressing HCC in rats by contrasting *H-Ras* targeted ASO therapy with a commercial formulation containing docetaxel (DXT). In order to determine the effectiveness and mechanistic intervention of the treatments, additional effort was made after obtaining a significant finding to use intravenous administration of the corresponding ASO in chemically induced HCC rats to inhibit mutated *H-Ras* or *c-raf.1* gene expressions frequently linked to HCC development and progression.

Antisense phosphorothioate-modified oligomer (PS-ASO) against the *H-Ras* gene (mutated) was physicochemically characterized, and its pharmacokinetic profile, hemolysis, liver uptake, distribution in kidney, lung, spleen, its effect in normal rats, and carcinogen-treated (HCC-induced) rats were investigated. These results were compared to DXT treatment in HCC rats. Immunohistochemical evaluation of CK-7, CD-15, Hep-par-I, p53 expressions, scanning electron microscopy assessment of liver tissue, levels of different liver marker-enzymes, and activities caspase-3 and caspase 9 were performed in the experimetal rats, along with in situ hybridization of mutated *H-Ras* expression. We then examined in vitro and in vivo investigations of *H-Ras* or *c-raf.1* gene expression by blocking them with the corresponding ASOs.

In comparison to DTX, PS-ASO exhibited a prolonged blood residence time in vivo, a time-dependent hepatic absorption, and minimal in vitro hemolysis (<3%). In healthy rats, it did not exhibit any harmful symptoms. Despite having a lower initial distribution in the lung compared to the liver and kidney, PS-ASO accumulated more in the lung than the kidney after eight hours. By suppressing *H-Ras* gene expression, certain immonohistochemical modulations, and

generating caspase-3/9-mediated apoptosis, PS-ASO (administered for 6 weeks) demonstrated superior antitumor potential in rats as compared to DTX in preventing chemically-induced carcinogenesis. In the experimental rats, it stopped lung metastatic tumors caused by HCC.

In human HCC cells, nanosize and well-dissociated ASOs that were administered against mutant *H-Ras* and *c-raf.1* exhibited varied IC50 values, whereas in normal human liver cells, they did not exhibit significant toxicity at the experimental dose range. In a healthy rat liver, labeled ASOs were evenly distributed. HepG2 and Huh7 cells treated with ASOs exhibited apoptosis and an increase of mitochondrial membrane potential. ASOs significantly reduced tumor occurrences and changed the number of localized lesions in the liver. In rat livers with HCC, ASO treatments decreased the expression of Hep Par I and HSP70 proteins, raised the expression of p53, and inhibited the expression of the corresponding genes. In HCC rats, ASO treatments changed the levels of the proteins caspase-3 and -9. Hepatic marker enzymes improved toward normal levels in rats treated with carcinogens and did not change when ASO was administered to normal rats.

PS-ASO (or ASO) was able to enter the experimental rat's liver tissues by a constant and prolonged release from the circulation. Furthermore, ASOs were efficiently distributed in the renal and lung tissues. It did not have any negative impacts on healthy rats. PS-ASO 6-week long therapy demonstrated significantly superior therapeutic efficacy than DTX in preventing the development of chemically produced tumors in rats. By boosting at least caspase-3 and caspase-9 activity (because these two caspases were examined in the current investigation), PS-ASOs showed therapeutic promise. Furthermore, PS-ASO decreased the likelihood that initial HCC would progress to a metastatic lung tumor. By using the in-situ hybridization approach, the HCC model created here demonstrated the presence of mutant *Ras* and *c-raf.1* genes. Apoptosis induction was affected differently by treatment with antisense oligonucleotides (PS-ASOs, also called ASOs occasionally here) that target either the mutant *Ras* or *Raf* gene. These effects included changes in mitochondrial membrane depolarization and a variety of cellular histochemical and biochemical parameters. Specifically, mutant *Ras* gene suppression produced by ASO shown a greater capacity to decrease HCC than *c-raf.1* gene inhibition. According to this study, PS-ASO may be able to modulate a number of proteins

linked to the development of HCC more broadly by targeting *H-ras* gene (mutated). Targeting the mutant *H-Ras* gene with PS-ASO therapy has great potential for treating HCC in the future and could be used to treat other cancers. In rats, PS-ASO mediated genetic therapy demonstrated the ability to prevent HCC much more successfully than DXT. Compared to ASO treatment against *c-raf.1*, ASO treatment against mutant *H-Ras* had superior therapeutic efficacy. Therefore, inhibiting mutant *H-Ras* rather than *c-raf.1* may have a significant impact on HCC inhibition in rats. More research is needed.

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Annexture

(Through the guide and Head of the Department of the Department of Pharmaceutical Technology, Jadavpur University)

To
The Registrar
Jadavpur University
Kolkata- 700032

Date : 19.04.2021

Subject- Joining in ICMR Senior Research Fellowship.

Dear Madam,

With due respect this is to state that I have received ICMR Senior Research Fellowship (copy of the letter attached) to work under the supervision of Prof. Biswajit Mukherjee on the project as mentioned in ICMR letter of Scholarship.

I am hereby joining from 19th April 2021 to the above mentioned fellowship program.

This is for your necessary action.

SS/Research
for n.o. pl
Registrar
23/4/2021

Yours sincerely,

Alankar Mukherjee
19.4.21

Alankar Mukherjee

Forwarded
19/04/2021
Professor (Dr.) Biswajit Mukherjee
Department of Pharmaceutical Technology
Jadavpur University
Kolkata-700 032, India

Accepted
23.6.21
Registrar
Jadavpur University

Forwarded
19.4.21
Head
Dept. of Pharmaceutical Technology
Jadavpur University
Kolkata-700 032, W.B. India

21/6/21
Research Section
Jadavpur University

যাদবপুর বিশ্ববিদ্যালয়

[redacted]
PRINCIPAL SECRETARY
FACULTY OF ENGINEERING & TECHNOLOGY

*JADAVPUR UNIVERSITY
KOLKATA-700 032, INDIA

Ref. No. D-A/E/371/22
Date: April 22, 2022

To
Smt. Alankar Mukherjee
Raipur, Aghore Sarani
Opposite to Paschatya Para More, "Sritani"
Kolkata, West Bengal
PIN – 700 149

Sub : Ph.D. (Engineering/ Pharmacy) Registration.

With reference to your application for registration to Ph.D. (**Engineering/ Pharmacy**) degree, you are hereby informed that you have been selected to register your name on payment of requisite Ph.D.Programme fee of **Rs.22000/-** (Rupees twenty two thousand only) payable in three semi annual installments (8,000/- + 8,000/- + 6,000/-). It may be noted that the date of payment of registration fee of the first installment shall be taken as the date of registration to the Ph. D. Programme & the Registration Certificate shall be issued to you by the university as per Ph.D regulation-2017 only after full payment of the Registration fee.

Registration shall remain valid for six years from the date of registration, provided that the candidate successfully completes the Ph. D. course work as stipulated in the Ph. D. regulation-2017 clause 20 (iii), he/she maintains regular contact with her/his Supervisor throughout the entire period of her /his work, her/his research progress is found to be satisfactory by the Research Advisory Committee (RAC) of the candidate concerned as per Ph. D. regulation – 2017 Clause 21(iii) and has paid registration fee and other fees applicable in full within 18 months from her/his date of registration.

He/She will have to satisfactorily complete Ph.D. course-work comprising at least two (2) courses of which one course shall be on Research Methodology and shall be of one semester duration. These courses must be taken within initial one or two semesters from the date of registration and must be successfully completed with at least 55% of marks in aggregate or its equivalent grade in the UGC 7-point scale and to be eligible to continue in the Ph.D programme and submit the thesis.

The proposed topic of research/ Title: **"Development of Antisense H-Ras Oligomers and Investigation of Their Potential as a New Drug Against Liver Cancer: In Vitro and In Vivo Studies".**

If the registration fee is not paid within a month from date of issue of this letter, your application may be treated as cancelled. Your research will be governed by this New Ph.D. Regulation-2017 **Please visit our University website www.jaduniv.edu.in for New Ph.D. Regulation-2017**. For any further clarification you may contact the office of the undersigned.

Subject to fulfillment of criteria and recommendation of R.A.C, the period of registration beyond Six years may be extended as per Ph.D. regulation-2017. Application for extension beyond Six years duly forwarded by the supervisor and recommended by the R.A.C. shall have to be submitted before expiry of Six years.

You are informing to bring the following documents at the time of deposition of Ph.D. programme fee.

1. Original Application Form.
2. Original NOC and Appointment Letter.
3. Photocopy of Ph.D. Registration Offer Letter.

(Samit Pahari)
Secretary, FET

Copy : Secretary, FET (Approved by Departmental Admission related Meeting dated 10.03.2022 and DC Meeting Dated 24.03.2022)
Records – 2
Supervisor/s – Prof./ Asst. Prof./ Dr. Tapan Kumar Giri
Department of Pharmaceutical Technology
Jadavpur University, Kolkata-700032

[KM]

Tel: 26588204-26588662-26589620

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भारतीय आयुर्विज्ञान अनुसंधान परिषद
INDIAN COUNCIL OF MEDICAL RESEARCH
वी. रामलिंगस्वामी भवन, अन्सारी नगर, पोस्ट बॉक्स 4911, नई दिल्ली - 110 029
V. RAMALINGASWAMI BHAWAN. ANSARI NAGAR. POST BOX 4911. NEW DELHI - 110 029

Date: 5.4.2021

No. 45/7/2020-/DDI/BMS

To,

Dr. Biswajit Mukhrerjee,
Profesdsor,
Pharmaceutics and Former Head,
Deptt. Of Pharmaceutical Technology,
Jadavpur University, Kolkata 700032

Subject:- Award of Senior Research Fellowship to Mr. Alankar Mukherjee on the research fellowship proposal entitled "Development of antisense H-ras oligomers and investigation of their potential as a new drug against liver cancer: in vitro and in vivo studies"

Sir/Madam,

The Director General, ICMR sanctions Senior Research Fellowship to Mr. Alankar Mukherjee, SRF on a stipend of Rs. 35,000/- p.m. to carry out research on the project mentioned above, under your guidance. H.R.A. and Medical reimbursement will be paid as per rules of your University/Centre.

The award of SRF will be subject to the following terms and condition:

TENURE: It will be tenable for from the date of joining duty and will be on yearly basis subject to maximum of Three years. .

Its continuance will, however, depend on the satisfactory progress of work and can be terminated at any time on a one month's notice, if the progress is not satisfactory, or on receiving adverse report from the Guide. The Fellow will be required to work on the project for a period at least one year.

The event of his/her leaving before completing one year on the fellowship, he/she may be required to refund the stipend drawn by him/her from the date of joining to the date of leaving the fellowship.

PRIVATE PRACTICE: Private practice of any kind, or taking up any appointment even in an honorary capacity during the fellowship is not permitted.

ADMINISTRATIVE CONTROL: The candidate will be under the administrative control of the Institution where he/she works, and will also be subject to the rules and regulations of the Institute.

LEAVE: Leave will be admissible according to the rules of the Institution, however in the case of female research fellows leave with stipend upto 6 months (in lieu of maternity leave) may be granted. No other kind of leave (such as sick leave) etc. will be admissible. Awardees are not entitled to vacation normally admissible to the staff of an Institution.

HRA: HRA will only be paid, if the fellow is not availing any hostel facility. A certificate to this effect should be sent along with joining report for payment of HRA.

REPORTS: The awardee shall submit 1st annual reports for the first 10 months on the prescribed standard proforma.

The first annual report should be submitted after 10 months from the date of commencement of the fellowship giving complete factual details of the research work done through the Guide alongwith his/her appraisal. Subsequent annual report should be submitted through the Guide two months before the completion of fellowship tenure. Failure to submit reports in time may lead to termination of the award.

Six copies of the final report in the prescribed form clearly shall be submitted one month before the date of termination of the award.

A list of the papers published or presented at Scientific Conferences during the tenure of the fellowship should also be furnished with the annual and final reports.

PUBLICATION OF PAPERS: Prior permission for publication of papers based on the research work done during the tenure of the award should be obtained from the Council. The paper should be sent to the Council through the Guide with his/her recommendations. Due acknowledgement to the Council should be made in these papers.

PAYMENT OF FUNDS: The stipend and the funds for contingencies shall be paid as per procedure laid down in the enclosed an annexure.

CONTINGENT EXPENDITURE: An annual contingent grant of Rs. 20000/- p.a. will be admissible. The contingent grant is given to meet petty expenditure for purchase of chemicals, reagents etc. No non-expenditure article or equipment can be purchased out of the grant.

TRAVEL:-

Traveling allowance will not be admissible for joining duty or on termination of the award.

The Council may approve tours of research fellows/associate for:-

1. **Attending symposium/seminar/conference provided the fellow/associate is presenting a paper which has been accepted by the organizers of the symposium/seminar/conference.**
2. **Field work connected with research**
3. **TA/DA would be admissible as per the rules applicable to Central Government Officers with basic pay equivalent to the amount of the fellowship stipend.**

NOTE:- The expenditure on this account will be met

POST FELOWSHIP CARRIER:-

1. The Research Fellow can register himself/herself for postgraduate qualification and to utilize in his/her the work done by him/her during his/her fellowship tenure. A copy of these submitted for postgraduate degree will have to be sent to the Council for information and record **from the contingent grant sanctioned to the fellow**. Due acknowledgement to the ICMR should be made in the thesis by the research fellows.
2. The Research Fellow should also send to the ICMR for information a brief report on the post/job taken by him/her after the expiry of the fellowship.

The date indication forenoon/afternoon on which he/she the fellowship may please be intimated to this office. He/she may be asked to report for duty within a month from the date of issue of this letter failing which the award will be treated as cancelled.

NOTE:

1. The fellow should submit the acceptance letter along with the joining letter clearly indicated forenoon/afternoon on which he/she joins the fellowship, forwarded through head of the institute stating that he/she will adhere to the terms and conditions of fellowships of ICMR. He/She may be asked to report for duty within a month from the date of issue of this letter failing which the award will be treated as cancelled.
2. Mandate form 9In original) to be types, signed and stamped on single page.
3. Cancelled cheque to be sent in original
4. Duly field HRA form should be sent in format enclosed.

Yours faithfully,

(G. S. Sandhu)
Sr. Administrative Officer
For Director-General

Copy to:-(Head of the Institution)

1. The Registrar, Jadavpur University, Kolkata 700032
2. Mr. Alankar Mukherjee, SRF, Deptt. Of Pharmaceutical Technology, Jadavpur University, Kolkata 7000342
3. Accounts Section – V, ICMR
4. IRIS Cell No. 2020-6927

Sr. Administrative Officer

No. FET 1368

Jadavpur University

Registration Certificate

Shri/Sm. Alankare Mukherjee

has been registered as a student of Ph.D. programme of this university

His/her Registration Number is 1022213004

Kolkata 10.05.2022 (Date of Reg.)

Registrar 06.01.23

JADAVPUR UNIVERSITY

KOLKATA-700 032

MARK SHEET

NO.: CW/19052/ 0828

(For Ph.D/M. Phil. Course Work)

Results of the PH.D. COURSE WORK EXAMINATION, 2023

In ENGINEERING & TECHNOLOGY

Name ALANKAR MUKHERJEE Class Roll No. 102211404012

Examination Roll No. PHDPHA23111

held in JANUARY, 2023

Subject Code / Name		Credit Hr.(c_i)	Marks
PHD/PH/S/121	RESEARCH METHODOLOGY	4	84
PG/PH/S/121	TERM PAPER LEADING TO THESIS	4	90

Total Marks : 174 (out of 200) **Remarks:** P

Prepared by : Checked by :

Date of issue : 04 / 04 / 2023

Controller of Examinations

Institutional Animal Ethics Committee (IAEC)

Department of Pharmaceutical Technology

Jadavpur University

Kolkata- 700032

Ref No: AEC/PHARM/1704/03/2020 Date: 05/03/2020

This is to certify that the project titled **"Development of Antisense H-Ras Oligomers and Investigation of Their Potential as a New Drug Against Liver Cancer: *In Vitro* and *In Vivo* Studies"** has been approved by the Institutional Animal Ethical Committee (IAEC), Jadavpur University in the meeting held on 3rd March, 2020.

05/03/2020

Prof. (Dr.) Biswajit Mukherjee
Chairman/Member Secretary IAEC

Dr. Kuladip Jana
CPCSEA Nominee

Chairman
Institutional Animal Ethics Committee
Jadavpur University
Kolkata-700032

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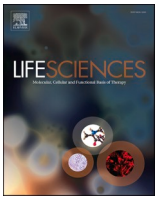

H-ras-targeted genetic therapy remarkably surpassed docetaxel treatment in inhibiting chemically induced hepatic tumors in rats

Alankar Mukherjee [a], Ramkrishna Sen [a,b], Ashique Al Hoque [a], Tapan Kumar Giri [a,*], Biswajit Mukherjee [a,*]

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ARTICLE INFO

Keywords:
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Antisense oligomer
H-ras
Hepatic lesion
Apoptosis

ABSTRACT

Aims: Hepatocellular carcinoma (HCC) is still a leading cause of cancer-related death worldwide. But its chemotherapeutic options are far from expectation. We here compared H-ras targeted genetic therapy to a commercial docetaxel formulation (DXT) in inhibiting HCC in rats.

Main methods: After the physicochemical characterization of phosphorothioate-antisense oligomer (PS-ASO) against H-ras mutated gene, the PS-ASO-mediated in vitro hemolysis, in vivo hepatic uptake, its pharmacokinetic profile, tissue distribution in some highly perfused organs, its effect in normal rats, antineoplastic efficacy in carcinogen-induced HCC in rats were evaluated and compared against DXT treatment. Mutated H-ras expression by in situ hybridization, hep-par-I, CK-7, CD-15, p53 expression patterns by immunohistochemical methods, scanning electron microscopic evaluation of hepatic architecture, various hepatic marker enzyme levels and caspase-3/9 apoptotic enzyme activities were also carried out in the experimental rats.

Key findings: PS-ASO showed low in vitro hemolysis (<3 %), and had a sustained PS-ASO blood residence time in vivo compared to DTX, with a time-dependent hepatic uptake. It showed no toxic manifestations in normal rats. PS-ASO distribution was although initially less in the lung than liver and kidney, but at 8 h it accumulated more in lung than kidney. Antineoplastic potential of PS-ASO (treated for 6 weeks) excelled in inhibiting chemically induced tumorigenesis compared to DTX in rats, by inhibiting H-ras gene expression, some immonohistochemical modulations, and inducing caspase-3/9–mediated apoptosis. It prevented HCC-mediated lung metastatic tumor in the experimental rats.

Significance: PS-ASO genetic therapy showed potential to inhibit HCC far more effectively than DXT in rats.

1. Introduction

Hepatocellular carcinoma (HCC), the most common primary liver cancer, is often acknowledged as one of the most difficult medical conditions to be managed therapeutically. HCC is the sixth most malignant cancer type and the fourth leading cancer death determinant worldwide [1,2]. The World Health Organization has predicted that both the new (diagnosed) cases and the death for liver cancer per year could exceed the figure approximately 1.4 million and 1.3 million, respectively, by 2040 [3].

HCC development is mostly asymptomatic at the early stages and has considerably long latent period [4]. Thus, the disease is usually diagnosed at the advanced stage [4], resulting chemotherapy as the only option for its therapeutic management. Many of the chemotherapies including multi-kinase inhibitors and immunomodulators [5] came with a great hope for HCC improvement with patient survival. However, clinical outcomes were far from expectation. Nearly all anticancer medicines are predominantly cytotoxic, low target specificity, poor solubility, inadequate penetrability in cancer cells/tissues, which develop chemotherapeutic resistance and HCC progression [6]. Nevertheless, chemotherapy is still the mainstay of HCC treatment.

Genetic therapies that include antisense oligonucleotides (ASOs) can inhibit oncoproteins, targeting mRNA or DNA precursors [7]. ASOs, the most promising class of short single stranded nucleotide chain-based therapeutics, have emerged as a potential therapeutic modality to address unrealized medical requirements. There have been six US-FDA (United States Food and Drug Administration)-approved ASO drugs in last seven years, and many are in the pipeline [8]. There has been an

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increasing interest in biological response modifiers in cancer therapy. ASO, typically 15–25 nucleotide single-stranded sequence targeted to specific messenger RNA, can inhibit the gene expression by interfering specifically with the translation of the corresponding protein at the mRNA level by Watson-Crick base pairing [9]. They are often modified chemically for their stability in biological fluids. Most common and widely used modification that increases its stability in blood and other body fluids is phosphorothioate (PS) backbone modification (one non-bridging oxygen is substituted with sulfur at the oligonucleotide backbone) [10]. PS-oligomers displayed significant metabolic stability and sequence specificity, causing absence of off-target effects [8]. During the last 6–7 years, ASO emerges as a new choice of modality for precision medicine and targeted therapeutics [11]. Enhanced nuclease resistance, low pro-inflammatory response, increased affinity and binding specificity to RNA with appreciable pharmacokinetic and pharmacodynamic features have edged ASO over other chemotherapies as an attractive choice [11].

Ras/raf/MAPK-signalling hyper-activation is a common observation in HCC [12,13]. H-ras gene, upon various mutations, undergoes genesis, progression and metastasis of HCC [14], and may be "a reliable marker" for HCC diagnosis and comprehensive HCC therapy [14,15]. However, how these H-ras mutations initiate cancer pathogenesis remains unclear. A mutation in codon 12 of H-ras that leads to a glycine to valine change, and consequently activates protein p21, in an irreversible guanosine 5′-triphosphate (GTP)-bound state, showing predominant oncogenic potential [16] widely recognized as hot spot in rodent hepatocarcinogenesis and human primary liver tumors [17].

In the present study, we have compared the efficacy of H-ras (mutated in codon 12)-targeted gene therapy to taxotere® (commercial docetaxel formulation) (DXT) in inhibiting carcinogen-induced HCC in rats. Several biochemical, histological, pharmacokinetic, histochemical, and morphological parameters were analyzed along with confocal and electron microscopic investigations to draw the inference.

2. Materials and methods

2.1. Materials

Designed phosphorothioated sequences (1–21):

5′-T*C*C*C*A*C*A*C*C*G*A*C*G*G*C*G*C*C*C*A*C-3′ with or without the 5′ modification with 6-carboxyfluorescein (6-FAM) (synthesized by Biotech desk, Hyderabad, India) were characterized and used for further study. Digoxigenin-labeled with dUTP and antisense 5′-U*C*C*C*A*C*A*C*C*G*A*C*G*G*C*G*C*C*C*A*C-3′ sequence and its complementary sense sequence, synthesized by Eurofins Genomics, Bangalore, India, were used for gene expression study. Commercial docetaxel formulation (Taxotere®) was procured from Aldine, Kolkata, India. All other chemicals used were either molecular biology grade or analytical grade.

2.2. Antisense oligomer characterizations

2.2.1. Mass spectroscopy

Mass spectrometry accurately determines the mass of a phosphorothioate oligomer [18]. We measured the mass of experimental oligomers, using the methods reported earlier [18,19]. After the ionization, the ions were separated by the mass-to-charge ratio (m/z) and then detected quantitatively.

2.2.2. Zeta potential

Two microliters of oligomer suspension was introduced into Millipore microbiology grade water, and briefly spinned. Subsequently, a volume of 1 ml was inserted into a quartz cuvette, and zeta potential was evaluated using Dynamic Light Scattering technique [20].

2.2.3. The atomic force microscopy (AFM)

The atomic force microscopy (AFM) was used to observe the morphology of labeled and unlabeled PS-ASOs and their three dimensional size. To prepare the PS-ASO sample, 50 μl of 1–10 μg/ml was deposited onto freshly cleaved mica and incubated for 10 min. For imaging in air, silicon cantilevers were used and the mica was then rinsed with buffer, dried with nitrogen, and placed in the instrument [21]. Once the images were captured, they were further processed with the help of Pico View software to obtain the final required stage.

2.2.4. Gel electrophoresis

The oligomer sample was kept in 4 °C and was denatured for 1 min at 95 °C and mixed with loading buffer, containing loading dye to put into the well. Agarose gel (2 %) was prepared by mixing 2 % agarose gel solution and ethidium bromide (was added in warm condition). It was placed on the casting tray for solidification. The comb was placed on it and kept it for rest for about 20 min for solidification and formation of gel. The gel apparatus was filled with 1× TAE (Tris base, acetic acid and EDTA) buffer along with the ladder and the sample was filled in the wells prepared by the comb. The gel was then run at a voltage 100 V for 45 min.

2.2.5. TEM analysis

The transmission electron microscope (TEM) is a technique to investigate internal morphology of a substance. PS-ASO suspended in Milli-Q water was dropped on carbon-coated copper grid (300-mesh), and air-dried. Images were obtained using a transmission electron microscope (JEOL JEM 2100 HR, Tokyo, Japan).

2.2.6. Selected area electron diffraction (SAED)

SAED is a popular crystallographic technique. The study was performed using a transmission electron microscope (JEOL JEM 2100 HR, Tokyo, Japan) [22]. Spot distribution obtained showed the SAED pattern.

2.2.7. In vitro hemolysis study of PS-ASO

In vitro hemolysis was studied following the method of Kumari et al. 2023 [21]. The blood collected from the tail vein into a heparinized tube was centrifuged at 4 °C for 5 min at 2000 ×g. The erythrocytes washed thrice with phosphate buffer-saline (PBS) (pH 7.4) after the removal of supernatant. A volume of 190 μl of erythrocyte suspension (2 %) in PBS was added to each well of a 96-well plate and treated with 10 μl of PS-ASO suspension of different concentrations, between 5 and 100 nM. They were then incubated at 37 °C for 12 h, with a gentle shaking. The erythrocytes undergone no hemolysis were collected by centrifugation (10,000 ×g for 5 min). The absorbance of the supernatant was estimated at 570 nm. The absorbance 100 % hemolytic blood sample was read. The percentage of hemolysis was calculated using the formula,

$$\textit{Hemolysis}\ (\%) = \frac{(\text{absorbance of the experimental sample} - \textit{a}\text{bsorbance of a solution with 0\%hemolysis})}{(\textit{a}\text{bsorbance of a solution with 100\%hemolysis} - \textit{a}\text{bsorbance of a solution with 0\%hemolysis})} \times 100$$

2.2.8. In vivo experiments

Male Sprague-Dawley (SD) rats were used for all the in vivo experiments. Upon receiving the approval of the University animal ethics committee (approval no.1704/03/2020), the animal experiments were carried out. Rats were housed six per cage at a constant temperature (22 ± 0.5 °C) and humidity (55 %) environment with a daily normal photo period.

2.2.9. In vivo hepatic accumulation of FAM-labeled PS-ASO

Rats of three groups each contained three male SD rats received FAM-labeled PS-ASO (2 mg/kg bodyweight) through the tail vein. The animals were sacrificed upon anesthesia. Liver samples were collected at 2, 6, and 8 h, and slide preparation was done without any additional dyes. The slides were observed through a confocal microscope.

2.2.10. In vivo plasma pharmacokinetic (PK) and tissue distribution study

Normal rats were divided into three groups each containing nine SD male rats. One group of animals was injected with FAM-labeled PS-ASO (2 mg/kg bodyweight) through the tail vein, and the other group of rats received Taxotere®, 2 mg/kg bodyweight, through the tail vein [20]. The third group (normal animals) received no treatment. Blood samples were collected at 2, 4, 6 and 8 h, and at the terminal experimental time point (8 h), approximately 1.0 ml blood was taken in a microcentrifuge tube by terminal heart puncture under anesthesia. The plasma was separated without any anticoagulant, followed by centrifugation at 3000 rpm for 6 min. The plasma samples were then read spectofluorimetrically [23], using normal plasma (from untreated normal rats) as control. The three animals from each group were then killed at 2, 6 and 8 h, and the liver, kidney, lungs and spleen were removed. A weighed portion of each tissue sample was homogenized in cold 1.15 % KCl, then sample was diluted and the PS-ASO level was determined spectofluorimetrically [23], using normal respective tissue homogenate as control. DTX was quantified as reported elsewhere [20]. Different PK parameters were then determined. Further, all FAM treated tissue samples of 8 h were processed, counter-stained with DAPI and evaluated by confocal microscopy [23].

2.2.11. Determination of treatment effect on normal and carcinogen-induced HCC rats

Hepatocarcinogenesis was developed in Sprague-Dawley male rats (initial bodyweight range of 100–120 g, 6–7 weeks old) with a continuous 16 week-long treatment of 0.05 % (*w*/w) 2-acytylamminofluorene (2AAF) as a complete carcinogen in their diet as reported earlier [24].

Animals were acclimatized to the facilities for 2 weeks before start of the experiment. They were housed six per cage in each group as mentioned below, at a constant temperature (22 ± 0.5 °C) and humidity (55 %) environment with a daily normal photo period. They received carcinogen-mixed diet in the morning at 10 am and the basal diet [24] at 3 pm, and water ad libitum. Animals were divided into the following groups, each containing 6 animals.

Group A- Normal untreated animals received only basal diet and water ad libitum.

Group B- Carcinogen control rats received 0.05 % (*w*/w) 2AAF in their diet for 16 consecutive weeks as a complete carcinogen, as mentioned above.

Group C- The rats received the same dose 2AAF in the diet for 16 weeks along with the treatment of PS-ASO (without FAM labeling) thrice a week for 3 weeks (from 31st week to 33rd week) at a dose of 2 mg/kg bodyweight, administered through tail vein.

Group D- The rats received the same dose of 2AAF in the diet for 16 weeks along with the treatment of PS-ASO (without FAM labeling), thrice a week for 6 weeks (from 31st week to 36th week) at a dose of 2 mg/kg bodyweight, administered through tail vein.

Group E- The rats received the same dose 2AAF in the diet for 16 weeks along with the treatment of commercial formulation (Taxotere®) thrice a week for 6 weeks (from 31st week to 36th week) at a dose of 2 mg/kg bodyweight [20], administered through tail vein.

Group F- The rats received the same treatment of antisense PS-ASO (without FAM labeling) thrice a week for 3 weeks along with the basal diet (without carcinogen) and water ad libitum.

Group G- The rats received the same treatment of antisense PS-ASO (without FAM labeling) thrice a week for 6 weeks along with the basal diet (without carcinogen) and water ad libitum.

Group H- The rats received the same treatment of the commercial formulation along with the basal diet (without carcinogen) thrice a week for 6 weeks and water ad libitum.

All animals were dissected after being anesthetized at the 38th week after the start of the experiment. All animals remained unfed for 12 h before being killed. All the liver samples, and those lungs that had tumors were taken out, examined macroscopically and processed for further analysis. No other organ had any visible tumor growth.

Each tissue sample was cut into small pieces. Hepatic samples of normal rats, and the normal rats that had received various treatments were evaluated for histological, enzymatic and SEM analyses. All other samples were used for histological, in-situ gene expression, immunohistochemical, SEM, and enzymatic analyses. Lungs that had tumors were undergone histological analysis only. Tissue samples were snap-frozen in liquid nitrogen, and were stored at −80 °C, until used further. The samples were thawed before use.

2.2.12. Macroscopic evaluation

Macroscopic examination of tissues collected from experimental rats was investigated for the presence of tumor growth or hyperplastic nodular growth, if any.

2.2.13. Microscopic evaluation

For histological studies, tissue fixation was done in 10 % formalin in PBS (pH 7.2). After processing the tissue, paraffin embedded tissue microtome sections of 6 μm were used for staining with toluidine blue (TB)/hematoxylin-eosin (HE)/and Periodic acid-Schiff (PAS), after the removal of the wax, and investigated under optical microscope. Various pre-neoplastic and neoplastic hepatic altered lesions were identified as hepatic altered foci (HAF). Numbers and sizes in terms of lesion area were determined by Zeiss light microscope and analyzed by Axio Vision software 4.7.1.

2.2.14. In-situ gene expression

H-ras mutated gene expression was studied by in-situ hybridization on the hepatic sections with the digoxigenin (DIG)-labeled antisense and sense oligomers of the gene in the carcinogen-treated rats and the rats treated with carcinogen and received the PS-ASO/DXT treatments, essentially by the method of Mukherjee et al. 2005 [10]. After the hybridization with the denatured probes at 37 °C for overnight, stringency washing was done with saline-sodium citrate buffer, pH 7, then using blocking 0.5 % blocking agent at room temperature. The slides were then incubated with anti-DIG-polyclonal antibody conjugated with alkaline phosphatase for 2 h. Then nitroblue tetrazolium/bromo-chloroindolylphosphate (NBT/BCIP) staining was done and observed under microscope.

2.2.15. Immunohistochemical evaluation

Immunohistochemical analyses were conducted employing the primary antibodies (anti-Hep Par 1, anti-CD15, anti-CK7, and anti-p53 antibodies) (Thermo-Scientific) along with their corresponding secondary antibodies, in accordance with the previously reported protocols [25–29]. After the incubation with the monoclonal antibody, the slides were washed and incubated with horseradishperoxidase-conjugated anti-mouse/anti-rabbit secondary antibodies (Dianova, Hamburg, Germany). The investigations facilitated the determination of specific proteins in the hepatic tissues.

2.2.16. SEM analysis

Liver tissue samples were obtained from normal, carcinogen control, and treated groups. The samples were fixed with 2.5 % glutaraldehyde in PBS (pH 7.2) for 1 h at 4 °C. The tissue samples were then prepared using a previously reported method [23] and subjected to examination under a scanning electron microscope (JSM Electron microscope, JEOL, Tokyo, Japan) at an operating voltage of 15 kV. All the samples were scanned at various magnification and photographs were taken.

The SEM hepatocellular tissue images were morphometrically analyzed for assessing various parameters such as area, perimeter, major axis, minor axis, and circularity, using ImageJ software.

2.2.17. Blood level of SGOT, SGPT, ALP

Blood samples were collected from the tail vein of rats and allowed to stand for 30 min without adding any anticoagulant. Then the samples were centrifuged at 2500 rpm for 10 min and the serum was collected and used for the analysis of serum glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT), and alkaline phosphatase (ALK) with a commercial kit (Coral Clinical Systems, Goa, India), following the manufacturer's protocol.

2.2.18. Study of change of bodyweight in rats

Bodyweight data were collected at the start of the experiment, and then on a weekly basis and the mean bodyweights were determined and presented here on every 5 week-basis, except the last set of those taken on the 36th week.

2.2.19. Caspase 3/9 activities

Hepatic thawed tissue samples were homogenized in cold 1.15 % KCl, then sample was diluted and the tissue homogenates were used for enzyme analysis. The activities of caspases 3, and 9 were determined as per the protocols, using a BioVision colorimetric caspase assay kit

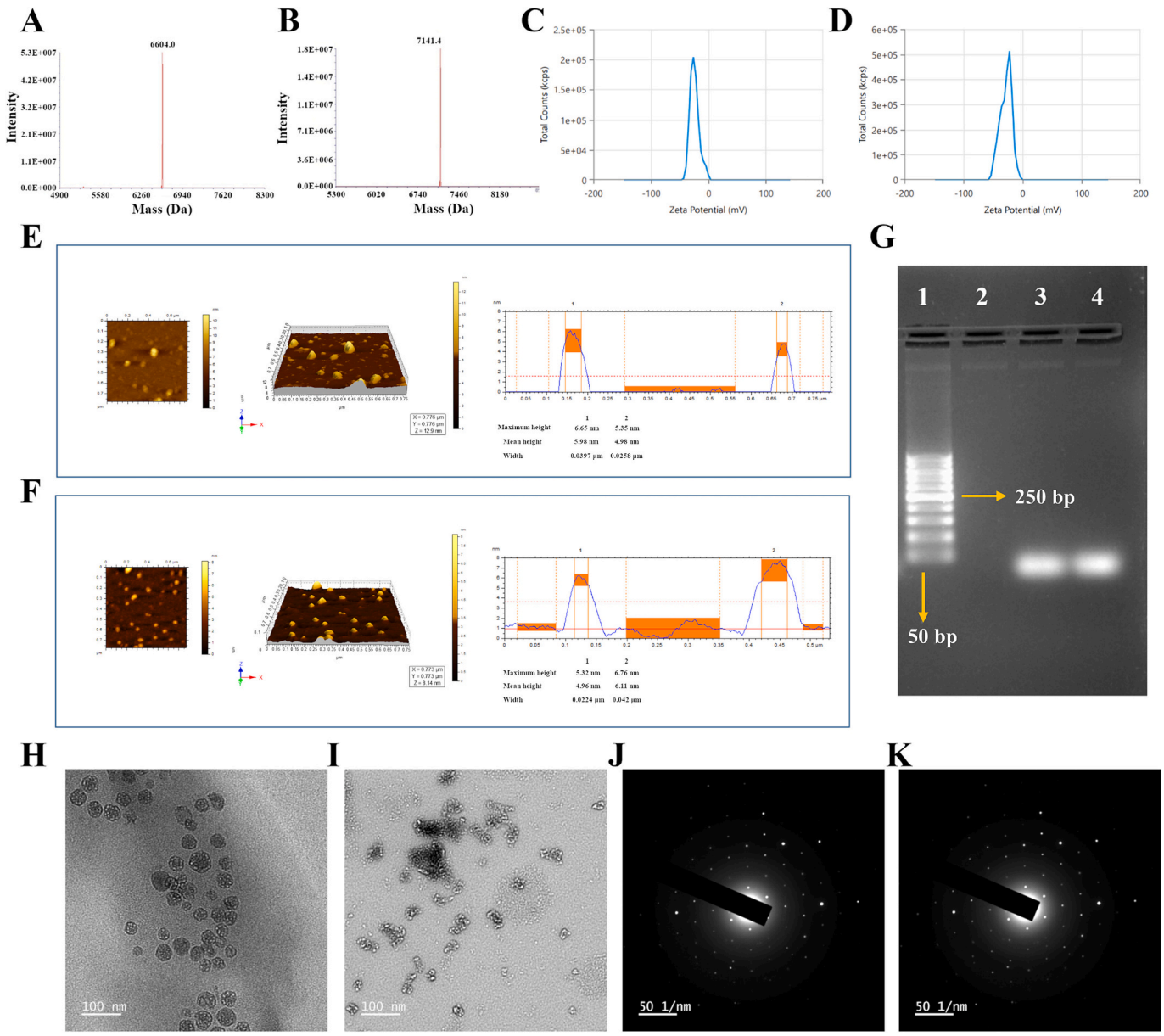


Fig. 1. Physicochemical characterization of FAM (fluorescein amidites)-labeled/unlabeled phophotothoate-backbone modified experimental antisense oligomer (PS-ASO). A. Mass spectrum of unlabeled PS-ASO; B. Mass spectrum of labeled PS-ASO; C. Zeta potential of unlabeled PS-ASO; D. Zeta potential of labeled PS-ASO; E. Atomic force microscopic data of unlabeled PS-ASO; F. Atomic force microscopic data of labeled PS-ASO; G. Gel electrophoresis data of unlabeled PS-ASO (lane 3), labeled PS-ASO (lane 4) and DNA ladder (Lane 1). Data show that both the PS-ASOs were 20 nucleotide-long; H. TEM microphotograph of unlabeled PS-ASO; I. TEM microphotograph of labeled PS-ASO; J. Selected area electron diffraction pattern (SAEDP) of unlabeled PS-ASO; K. SAEDP of labeled PS-ASO.

(BioVision, Milpitas, California, USA).

2.2.20. Statistics

All data obtained were analyzed by one-way ANOVA followed by a post-hoc test, Student's *t*-test or Dunnett test (Dunnett's *t*-test), using GraphPad Instat version 3.0 (GraphPad Software, Inc., San Diego California, USA).

3. Results

3.1. Physicochemical characterization data of antisense oligomers

Antisense oligomer, with or without FAM-labeled single-stranded DNA sequence of 21 nucleotides, was designed against H-ras mRNA containing a homogeneous point mutation at codon 12. The mass spectra of oligomers (Fig. 1 A, B) showed that molecular weight (g/mol) of the unlabeled oligomer was 6604.0 and that with FAM label was 7141.4 g/mol. FAM incorporation enhanced the mass of oligomer. Zetasizer analysis showed that FAM conjugation decreased zeta potential from −24.03 mV to −28.21 mV (Fig. 1 C, D). The AFM data (Fig. 1 E, F) suggest that that there was no predominant variation between FAM labeled and unlabeled PS-ASOs. PS-ASOs had a mean height of 5.48 nm, and width of 32.75 nm, and the labeled PS-ASOs had the values 5.53 and 32.2 nm, respectively. The height slightly increased with FAM labeled PS-ASOs. A recent study also reported that FAM conjugation led to an enhanced height and decreased surface charge [30]. Gel electrophoresis

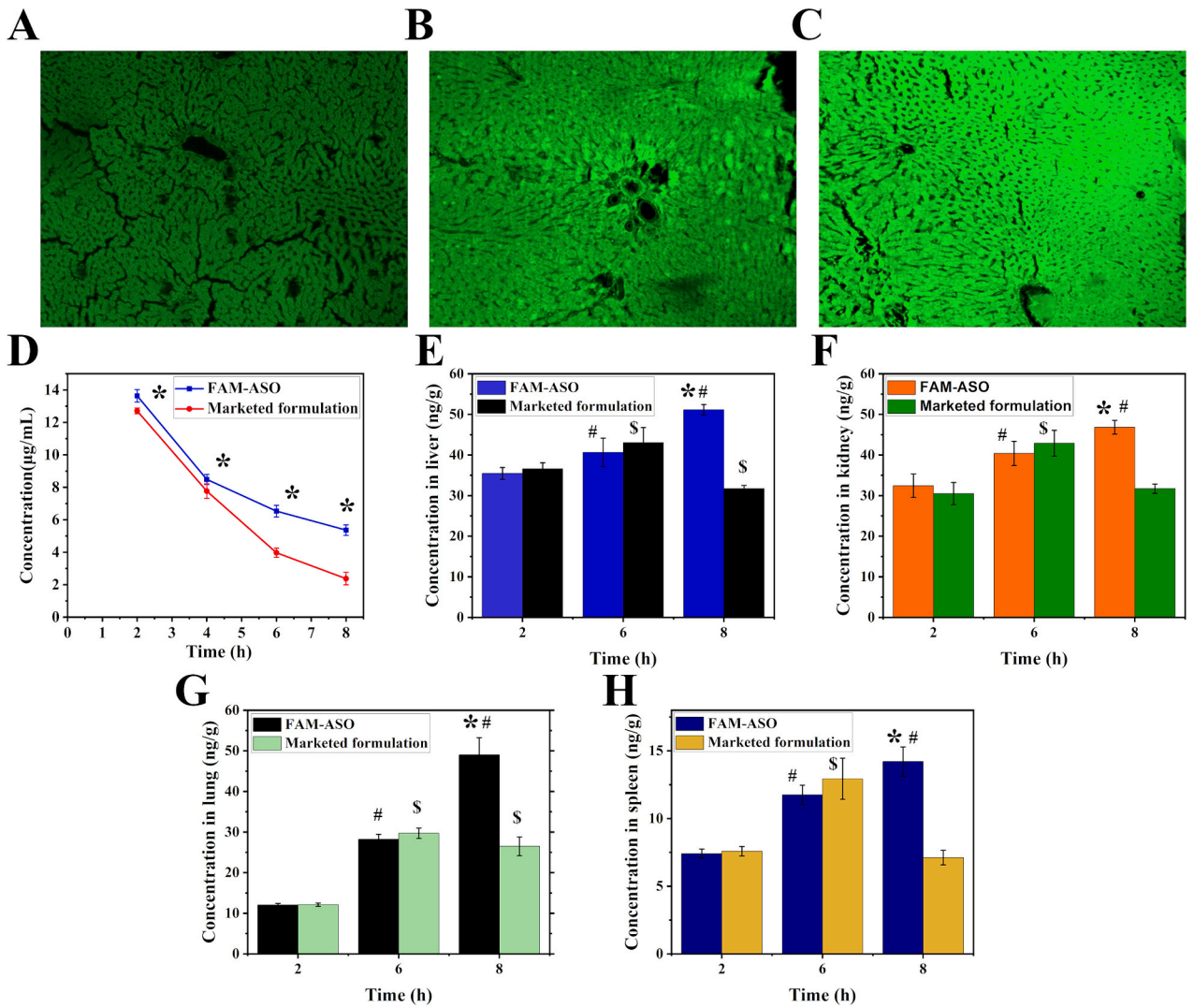


Fig. 2. Hepatic accumulation, blood level, and few highly perfused tissue distribution patterns of FAM-labeled PS-ASO (FAM-ASO) administered through the tail vein of normal rats. A. Confocal microscopic photograph of liver, 2 h after FAM-ASO administration; B. Confocal microscopic photograph of liver, 6 h after FAM-ASO administration; C. Confocal microscopic photograph of liver, 8 h after FAM-ASO administration; D. Blood levels FAM-ASO/docetaxel at different time points after the intravenous administration of FAM-ASO/docetaxel formulation (DXT). Data show mean± standard deviation ($n = 3$); *$p < 0.05$ is the statistical level of significance when compared against DXT treated blood levels. Data were analyzed using one-way ANOVA followed by Student's *t*-test. E. Liver concentrations of FAM-ASO/docetaxel at the different time points after the intravenous administration of FAM-ASO/docetaxel formulation (DXT). F. Kidney concentrations of FAM-ASO/docetaxel at the different time points after the intravenous administration of FAM-ASO/docetaxel formulation (DXT). G. Lung concentrations of FAM-ASO/docetaxel at the different time points after the intravenous administration of FAM-ASO/docetaxel formulation (DXT). H. Spleen concentrations of FAM-ASO/docetaxel at the different time points after the intravenous administration of FAM-ASO/docetaxel formulation (DXT). Data show mean±standard deviation (n = 3); *$p < 0.05$ is the statistical level of significance when compared against DXT treated tissue level at 8 h. $^{\#}p < 0.05$ is the statistical level of significance when compared against FAM-ASO treated tissue level at 2 h. $^{\$}p < 0.05$ is the statistical level of significance when compared against DXT treated tissue level at 2 h. Data were analyzed using one-way ANOVA followed by Student's *t*-test for the data marked with (*) at 8 h compared against respective DXT concentrations, and by Dunnett's *t*-test for the comparison with the data (marked with $^{\$}$, $^{\#}$) of FAM-ASO/DXT against their values at 2 h.

data suggest that PS-ASO lengths were nearly same of 21 nucleotides for both (Fig. 1 G). TEM analysis shows that PS-ASO appeared as particles around 40 nm in diameter with nearly globular drop-shaped subunits and the density distribution in each subunit was not completely symmetric (Fig. 1 H, I). Each blob was associated with one of its neighbouring subunits. Many spaces (white spots) were seen and the structure seemed to be spiral. This suggests for an existence of a vorticity in the PS-ASO structure. Selected area electron diffraction pattern (SAEDP) study shows that many discrete spots (atoms) lined up and formed rings (Fig. 1 J, K). Polycrystalline structures provide such ring structures in SAEDP. The small spots arose from Bragg's reflection from many small discrete crystals. Our data support a polycrystalline structure of macromolecular architecture for PS-ASO.

3.2. PS-ASO produced low in vitro hemolysis

PS-ASOs have been well-reported to be stable in blood by several studies [31,32]. In vitro hemolysis study conducted aseptically for 12 h in blood samples showed low hemolysis (<3 %) for PS-ASO and 3.45 % for DTX (Supplementary Table S1).

3.3. Findings of animal experiments

3.3.1. Prolonged retention of PS-ASO in liver

Initially, we investigated whether the FAM-labeled antisense oligomers (PS-ASO) injected through tail vein of normal rats reaching liver. Confocal microscopic study confirms that AFM labeled antisense oligomer reached well in the liver (Fig. 2 A–C) and remained in the liver well even after 8 h of the injection (the maximum period of this study conducted). The figure showed increased green fluorescence of FAM-labeled PS-ASOs with the increasing time within the hepatic tissue. The data suggest that longer presence of PS-ASO in liver and thus, it could work for a longer period.

3.3.2. Enhanced blood residence time and increased liver, kidney and lung accumulation of PS-ASO than DTX in the normal rats

In the dose escalation study, 0.5 mg/kg dose was determined initially as the minimum effective dose in 50 % rats (ED50) based on 25 % caspase 3/9 activity induction capabilities in the liver of normal adult Sprague-Dawley rats (6–7 weeks old male). The experiment was done on 6 normal adult rats in each group of three groups, with variable doses of 0.25 mg/kg, 0.5 mg/kg and 1.0 mg/kg bodyweight, respectively. Once ED50 (0.5 mg/kg bodyweight) was obtained, another set of experiment was performed. The experiment was done by taking 4 normal adult male rats (6–7 weeks old) for each dose. The dose was enhanced from 0.5 mg/kg to 1 mg/kg bodyweight, and then with an addition of 1 mg to each escalation of dose (such as 1, 2, 3, 4, 5, …mg/kg bodyweight) to determine TD50 (toxic dose where 50 % animals showed toxicity, but without any death). Fatigue in animals with 25 % enhancement of blood ALK, SGOT and SGPT was considered as dose-related toxicity. First animal showed fatigue, but with <25 % increased enzyme levels at 4 mg/kg bodyweight dose. Next fatigue with 45–60 % enhancement of the enzyme levels was seen in 2 rats at 11 mg/kg bodyweight dose. Finally, at 12 mg/kg bodyweight dose, 50 % of animals showed fatigue with 58–76 % increase in the enzyme levels. Hence, 12 mg/kg bodyweight dose considered as TD50. Therapeutic index (TI) was determined using the formula, ID = TD50/ED50, and the value was 24 mg/kg bodyweight. Since fatigue was observed at 4 mg/kg dose in a rat, we selected here a safer dose of unlabeled PS-ASO of 2 mg/kg bodyweight or equivalent for labeled PS-ASO. However, the dose we used for the investigation was found to be safe as observed from the findings of PS-ASO-treated normal animals.

Then we have performed pharmacokinetic and biodistribution study of the FAM-labeled PS-antisense oligomers injected through tail vein of rats. PS-ASOs are known to be very stable in blood [31,32]. DTX levels in blood were also determined after the intravenous administration of Taxotere® in the tail vein of rats. Pharmacokinetic profile (Supplementary Table S1) and biodistribution of Taxotere® (Fig. 2) were also determined. Labeled PS-ASO concentrations in blood and some perfused tissues were assessed using spectrofluorimeter. Various pharmacokinetic parameters were determined.

Blood level of PS-ASOs dropped slowly from 2 to 8 h (study period) compared to DTX from the equivalent Taxotere® dose, which showed faster release of DTX than PS-ASO from blood (Fig. 2 D). PS-ASO showed more sustained drug level in blood, suggesting a prolonged therapeutic action. The half-lives were determined to be 4.8 h and 6.4 h for DTX and PS-ASO, respectively. The bioavailability till first 8 h, $(AUC)_{0\text{-}8h}$, and the average operative body clearance values [33] were 67.5 $\mu g \cdot h \cdot ml^{-1}$ and 84.2 $\mu g \cdot h \cdot ml^{-1}$, 0.0059 $l \cdot h^{-1}$ and 0.0047 $l \cdot h^{-1}$, respectively (Supplementary Table S1).

Variable tissue PS-ASO concentrations were observed (Fig. 2 E–H). PS-ASOs were distributed rapidly in the liver and kidney within first 2 h, and eventually the levels were increased at 6 h and 8 h (our total study period for this study). PS-ASO concentration was initially (at 2 h) much less in lungs and spleen. However, PS-ASO concentration in lungs increased sharply at 6 h and 8 h. At 8 h, PS-ASO level in lungs was nearly closed to those values in liver and kidneys. In fact, the levels of PS-ASO concentrations in these three tissues were in the order of liver > lungs > kidneys at 8 h. The tissue sections were also observed with confocal microscopy (Fig. 3). The investigation also supports the findings of PS-ASO tissue levels as quantified spectrofluorimeterically. Further, very low PS-ASO concentration was undetectable in spleen by confocal microscopy. DTX tissue levels were also determined after the intravenous administration of Taxotere® in the tail vein rats. DTX tissue levels were found to be maximum at 6 h and then the DTX levels were decreased in the investigated tissues. The data suggest that PS-ASO levels in blood

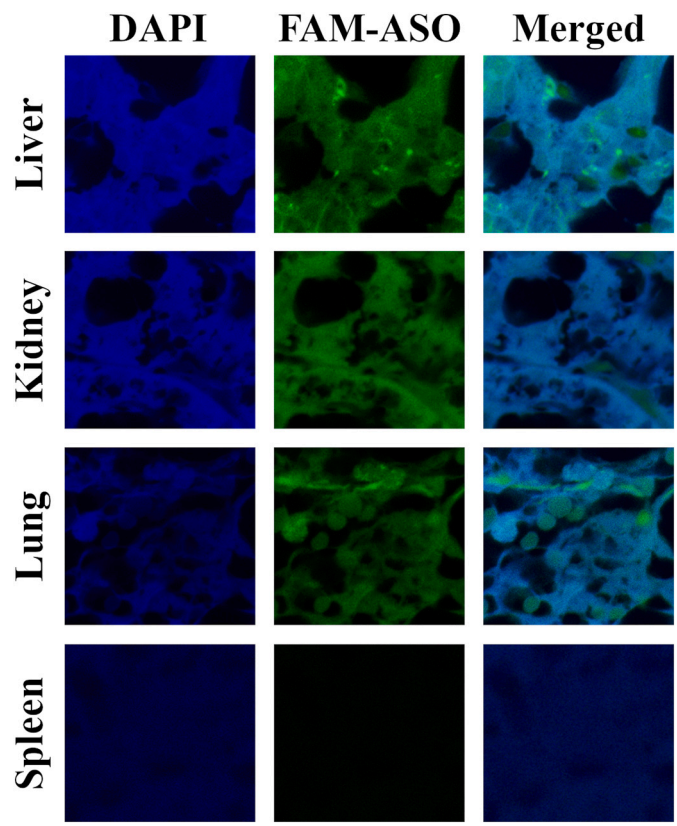


Fig. 3. Confocal microscopic photograph (60×) of some highly perfused organs at 8 h after the FAM-labeled PS-ASO (FAM-ASO) administration through the tail vein of normal rats. Counter-stained with DAPI showed blue colour, FAM gave green fluorescence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

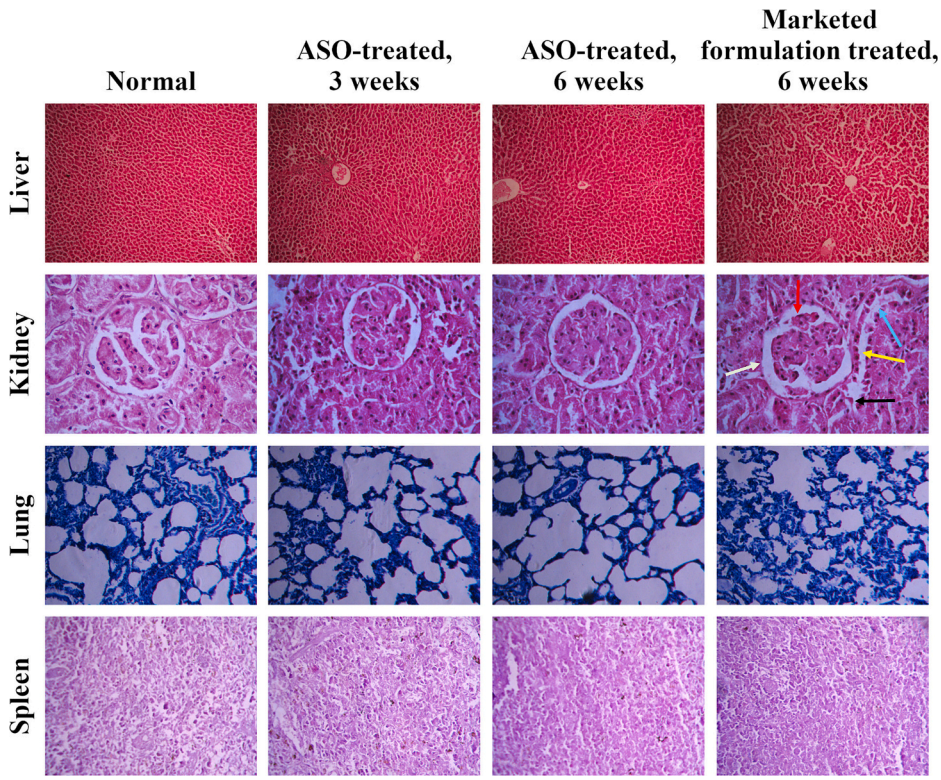


Fig. 4. Histological microscopic photographs of liver (10×), kidney (40×), lung (40×) and spleen (40×) of normal (untreated) rats and normal rats received experimental treatments. PS-ASO here represented as ASO in the figure. Renal histological changes are shown by differently coloured arrow heads; tubular atrophy (shown by sky blue arrow head), interstitial fibrosis (shown by black arrow head), tubular dilation (shown by yellow arrow head), dilated Bowman capsule (shown by off-white arrow head), glomerular atrophy (shown by red arrow head). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and the tissues achieved after its administration varied distinctively from those of DTX administration.

3.3.3. PS-ASO treatments showed no notable toxic effect in liver, kidney, lung and spleen of normal rats

We have also investigated effect of PS-ASO/DTX administration in some highly perfused organs such as liver, kidney, lung, and spleen in normal rats. DTX administration brought some toxic manifestations with some morphological alterations such as enhanced sinusoidal spaces in hepatic tissues (Fig. 4). A marked thickening of interalveolar septa was seen in the lungs of DTX-treated rats. PS-ASO administration for 3 weeks, and the 6-week treatment showed no sign of morphological abnormality as seen in the case of normal tissues. DTX treated rats showed notable toxic effect in the organs tested here. Microscopic examination of histological renal tissues of Toxotere®-treated rats showed tubular atrophy, interstitial fibrosis, tubular dilation, dilated Bowman capsule, and glomerular atrophy. Data suggest that normal rats treated with PS-ASO for 3/6 weeks did not show any predominant toxic manifestations as observed through morphological alteration.

3.4. In vivo antineoplastic effects of PS-ASO/DTX treatment

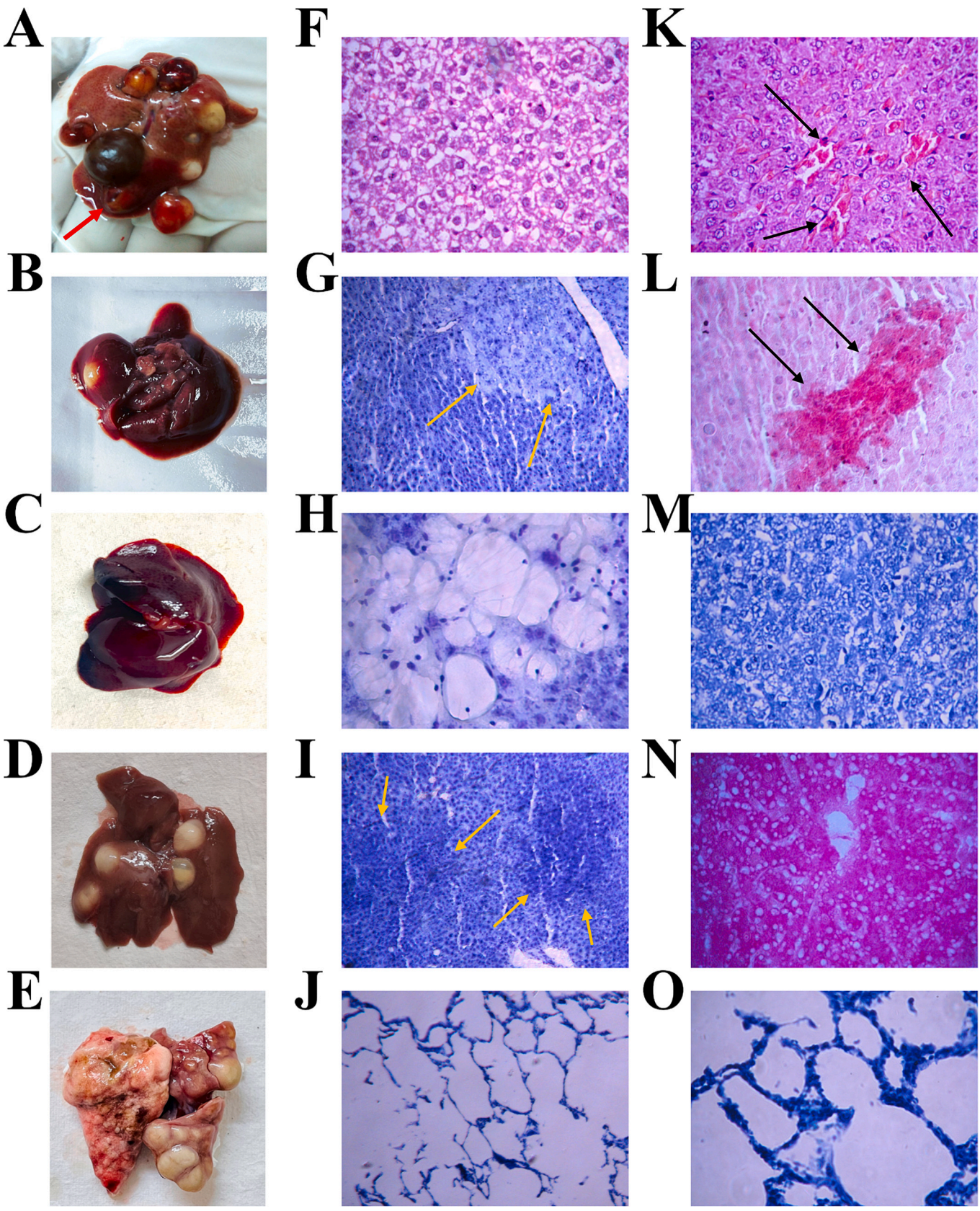
3.4.1. PS-ASO notably reduced hepatic tumor incidences

Macroscopic examination showed distinctive tumor growth in carcinogen control animals (Fig. 5 A–D). Grey-white hyperplastic nodules eventually turned to grey to red tumor masses to large tumors of hepatocellular carcinoma. Dark tumor even bulged out from the liver by cracking liver surface (Fig. 5 A). Further, carcinogen control animals (two out of six animals) even showed hepatic metastasized tumor in lungs (Fig. 5 E). However, PS-ASO-treated carcinogenic animals showed greyish white hyperplastic tumor nodules upon receiving 3-week-long PS-ASO treatment (Fig. 5 B). However, 66 % reduction in tumor incidences was seen in carcinogen-treated rats received PS-ASO treatment for 3 weeks (Table 1). PS-ASO treatment for 6 weeks showed disappearance of hyperplastic nodules in the liver of the carcinogen treated rats.

Taxotere® administration also showed tumor reduction. But tumor nodules were more in Taxotere® treated rats than those seen in rats received PS-ASO treatment for 3 weeks and 6 weeks.

3.4.2. PS-ASO treatment reduced precancerous and cancerous hepatic altered focal lesions and inhibited lung metastasis

Microscopic examination showed that hepatic altered focal lesions (HAFs) were predominantly scattered throughout the non-tumor hepatic areas of carcinogen control rats. There were large areas of tumor occupancy in the livers of those animals. HAFs such as ground-glass lesions, mixed cell focal lesions, basophilic lesions, spongiosis hepatis, angiectatic lesions, hypereosinophilic (preapoptotic) lesions etc., were predominantly seen in the carcinogen control rat livers (Fig. 5 F–N). Preneoplastic ground-glass lesions and basophilic lesions were well-associated with HCC development in the cellular lineage of glycogen storage (ground-glass) lesions (Fig. 5 F), to mixed cell lesions (eventually ribosome-rich and glycogen-poor) (Fig. 5 G). Here we also demonstrated spongiosis hepatis (Fig. 5 H) formed by distended hepatic stellate cells and ribosome-rich basophilic lesions (Fig. 5 I), which ultimately lead to HCC in the liver of carcinogen control rats. In our present study, we also observed that such lesions were varied with large to smaller sizes based on the treatments received by carcinogen treated rats. PS-ASO treatment for 6 weeks in carcinogen-treated rats showed a maximum reduction of large lesions (>3 mm), but increased number of small preneoplastic lesions (<1 mm) in livers (Table 1). Spongiosis hepatis, a multilocular cystic degeneration, are formed by distended hepatic stellate cells and



(caption on next page)

Fig. 5. Macroscopic and microscopic liver and metastatic lung images of carcinogen control rats, and rats treated with carcinogen and received the experimental treatments. PS-ASO here represented as ASO in the fig. A. A macroscopic liver image of carcinogen-induced HCC rats. The photograph shows multiple tumors and grey-white hyperplastic tumor nodules, and a tumor bulged out, cracking the liver surface (shown by a red arrow head). B. A macroscopic liver image of carcinogen-induced HCC rats received PS-ASO-treatment for 3 weeks. C. A macroscopic liver image of carcinogen-induced HCC rats received PS-ASO-treatment for 6 weeks. D. A macroscopic liver image of carcinogen-induced HCC rats received docetaxel formulation (DXT) for 6 weeks. E. A macroscopic image of carcinogen-induced HCC rat lung, showing metastatic tumor development. Various preneoplastic and neoplastic hepatic lesion images (F–I, K–N) (40×) and microscopic lung images (J and O) (60×) in carcinogen-induced HCC rats. F. Ground glass lesion; G. Mixed cell focal lesion (shown by yellow arrow heads), upon TB staining; H. Spongiosis hepatis, showing distended hepatic stellate cells, upon PAS staining; I. Darker blue basophilic lesions (shown by yellow arrow heads), upon TB staining; J. Alveoli structure in tumor area, showing emphysema, upon TB staining; K. Angiectasis lesion (Peliosis hepatis), with increased sinusoidal space and filled with blood cells (shown by black arrow heads), upon H&E staining; L. Hypereosinophilic lesion (shown by black arrow heads), upon H&E staining; M. Carcinoma in the hepatocytes, upon TB staining; N. Fatty liver, where fat droplets appeared as clear-hollow in the hepatocyte upon H&E staining; O. Alveoli structure in non-tumor area, upon TB staining. H&E, Hematoxylin-eosin, TB, toluidine blue, PAS, periodic acid-Schiff. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Tumor incidences, and sizes of hepatic altered focal lesions (HAFs) in experimental rats.

Animal groups	Tumor incidences (out of six animals in each group)	Incidences of (%) hepatic altered focal lesions (HAF)/cm^2 hepatic tissue area		
		<1 mm	1 mm–3 mm	>3 mm
Group A (normal control)	0/6			
Group B (carcinogen control)	6/6	15±2.1	32±2.8*	53±3.8*
Group C (carcinogen-treated rats treated with ASO for 3 weeks)	2/6	46±2.6#	33±2.2*,#	21±1.8*,#
Group D (carcinogen-treated rats treated with ASO for 6 weeks)	0/6	81±3.4#	14±2.0*,#	05±1.2*,#
Group E (carcinogen-treated rats treated with Taxotere® for 6 weeks)	4/6	32±1.8#	38±2.2*,#	30±1.4*,#
Group F (normal rats treated with ASO, as ASO control)	0/6			

Data show mean±SD ($n = 6$); Data were analyzed by two way ANOVA, followed by Dunnett's test.
* $p < 0.05$, when alteration of HAF incidences was compared with those of the size (<1 mm).
# $p < 0.05$, when alteration of HAF incidences was compared with those of Group B rats.

has flocculent eosinophilic cytoplasm. They are often seen in liver developing HCC. Metastatic tumor in lung showed emphysema in the tumor area (Fig. 5 J). It has been discussed later. Angiectasis lesions (Peliosis hepatis) were seen in the carcinogen control rats. The type of lesions occurred due to the atrophy of neighbouring hepatocytes, often with increased sinusoidal spaces and filled with blood cells (Fig. 5 K). They are associated with HCC development and progression. Treatments reduced the occurrence of apoptotic hepatocytes, and maximum reduction was observed in PS-ASO treatment for 6 weeks. Apoptotic hepatocytes became hyper eosinophilic with condensed and often with fragmented nucleus materials. Nuclear chromatid condensations, nucleus membrane blebbing, formation of apoptotic body and polypoidal hepatocytes with multiple nuclei or a single large nucleus were also seen. Eosinophilic lesions are often multifocal, usually small eosinophilic granular lesions with irregular periphery seen more in the early stage of carcinogenesis (Fig. 5 L). Hepatic carcinoma was predominantly seen in the case of carcinogen control rats (Fig. 5 M). Cytoplasm of those dying hepatocytes often had clear hollow due to fragmentation or cellular shrinkage. Fat deposition in the cytoplasm of the hepatocytes of carcinogen control rats was also seen. Accumulated lipids provided a clear appearance such as vacuoles in the hepatocytes with H&E staining (Fig. 5 N). Numbers and sizes of HAFs were remarkably reduced in carcinogen treated animals received PS-ASO treatment (Table 1). PS-ASO treatment for 6 weeks showed maximum reduction of HAF both in size and in numbers compared to its 3 week-long treatment/DTX treatment in the carcinogen treated rats. PS-ASO treatment for 6 weeks showed minimum incidence (10 %) large HAF (>3 mm), whereas, maximum small lesion (<1 mm), suggesting delaying the progress of carcinogenesis compared to PS-ASO treatment for 3 weeks. Carcinogen control rats showed maximum incidence (53 %) of large HAF (>3 mm) with a minimum number of small HAF (<1 mm). Normal rats and normal rats-treated with PS-ASO for 6 weeks maintained to normal hepatic architecture. Taxotere® treated carcinogenic rats showed predominantly large HAF (>3 mm) compared to those PS-ASO-treated carcinogenic rats, but the value was less than the carcinogen control rats. The small HAF lesions (<1 mm) were less in Taxotere® treated rats than those of carcinogen-treated rats, but were more compared to the small HAF lesions (<1 mm) of PS-ASO-treated rats. Intermediate lesion types (1–3 mm) had similar trend to small HAF lesions (<1 mm) for Taxotere® treated rats.

Lungs showed hepatic metastatic tumors in some rats (Fig. 5 E). Emphysema was predominantly seen in the metastatic lung tissues with enlargement of alveoli, thinner alveolar walls, and breakdown of many alveolar walls. This caused many larger air spaces in place of many small usual alveoli, causing damaging of lungs (Fig. 5 J). The thickness of alveolar walls in non-tumor areas looked normal (Fig. 5 O).

Hepatic altered focal lesions (HAF) were shown in a larger tissue area (magnification 10×) of the hepatic sections of the experimental rats (Fig. 6), for better understandings and clarification for their distribution patterns. Large ground glass lesion was predominantly visible in carcinogen control rats. The lesion type was less prominent and smaller in size upon receiving the experimental treatments. However, the lesions were most effectively reduced both in numbers and sizes upon PS-ASO treatment for 6 weeks. Similar findings were noted with the other lesions such as basophilic, mixed cell, hyper eosinophilic and angiectasis lesions.

3.4.3. PS-ASO successfully inhibited mutated H-ras gene expression in the liver of carcinogen-treated rats

In the present study, in situ hybridization at cellular level using digoxigenin-labeled antisense oligomers showed that PS-ASO-treatment successfully inhibited H-ras gene expression that had overexpressed predominantly in the liver of carcinogen control rats (Fig. 7 A) (Supplementary Table S2). Quantification of the data of gene expression by in situ hybridization and the various protein expression by immunohistochemical method was done by combined semi-quantitative scoring analysis [34–36]. PS-ASO treatment for 6 weeks showed superior effect to 3-week PS-ASO treatment, in inhibiting the mutated H-ras gene expression. The data suggest that the efficacy of the selected sequence of PS-ASO remarkably inhibited mutated H-ras gene expression at the cellular level. Sense control data showed no mutated H-ras gene expression, suggesting accuracy of blocking the mutated H-ras gene expression by PS-ASO. Taxotere® administration showed a moderate reduction of H-ras gene expression in hepatic tissues of the carcinogen-

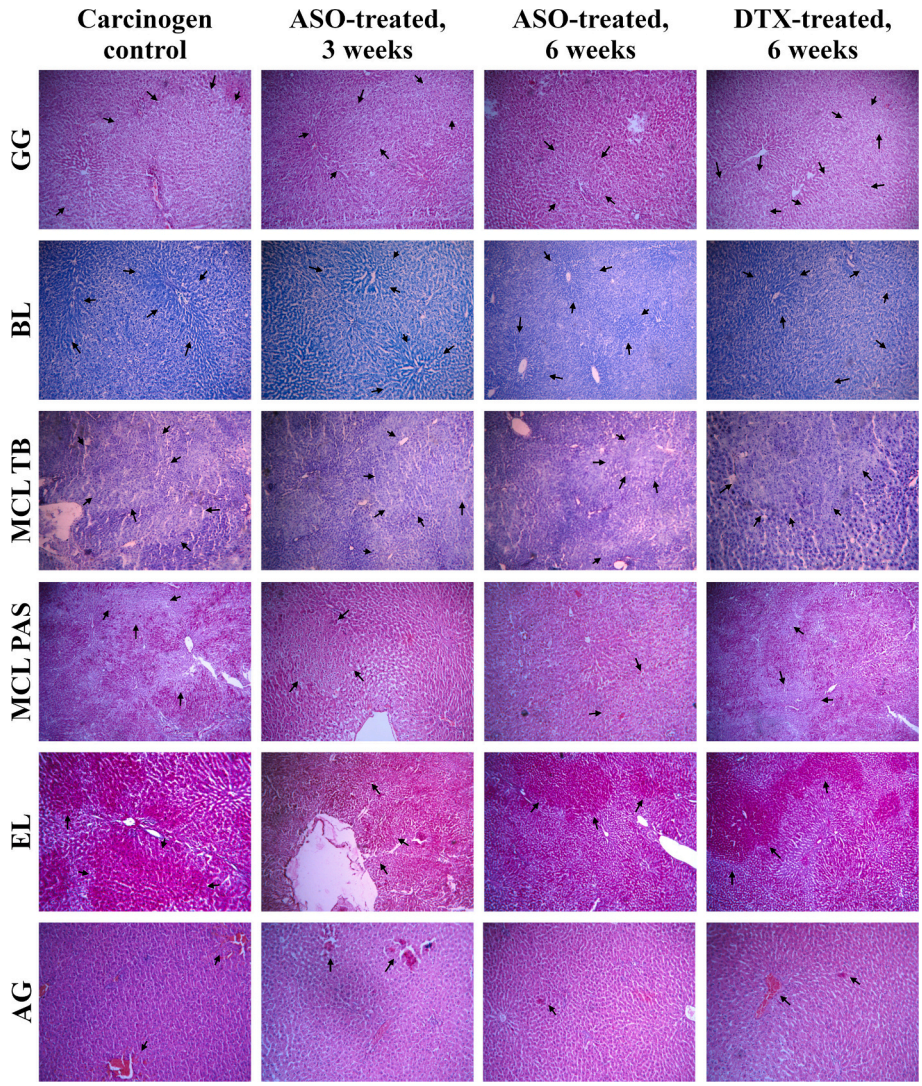


Fig. 6. Various hepatic altered focal lesions in larger hepatic areas (10×) of the experimental rats. Lesions were shown by arrow heads. GG, ground glass lesions; BL, basophilic lesions; MCL TB, mixed cell lesions with toluidine blue staining; MCL PAS, mixed cell lesions with periodic acid-Schiff staining; EL, eosinophilic lesions; AG, angiectasis lesions. PS-ASO here represented as ASO in the figure. DXT, docetaxel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treated rats.

3.4.4. Variable hepatic expression patterns of Hep par-1, CK-7, CD-15, and p53 in the experimental rats

Immunomarkers have been well recognized for selective labeling of poorly differentiated and well-differentiated HCC. In immunohistochemical investigation, we have selected some widely accepted immunohistochemical HCC markers such as Hep par-1, CK-7, CD-15, and p53. We investigated the Hep par-1 protein expression in the experimental tissue sections by immunohistochemistry (Fig. 7 B). Carcinogen control rats showed highest level of expression of the Hep par-1 protein. PS-ASO treatment reduced the protein expression. Expression was lower with longer treatment (6 weeks) of PS-ASO (Supplementary Table S2). Normal rats and normal rats treated with PS-ASO did not show the expression of the protein. Carcinogen-treated rats received Taxotere® showed more or less similar level of hepatic Hep par-1 protein expression in hepatic tissues to PS-ASO treated (for 3 weeks) rats treated with carcinogen. CK-7 and CD-15 expressed in carcinogen treated rats, and the experimental treatment-mediated inhibition of CK-7 and CD-15 proteins suggests that the treatments had the ability to reduce the level of the CK-7 and CD-15 expressions in the livers of rats received carcinogen (Fig. 7 B). Long-term (6 weeks) PS-ASO treatment inhibited the protein expression to a greater extent compared to PS-ASO (for 3 weeks) and Taxotere® treatments. However, p53 expression was minimal in carcinogen control rat livers (Fig. 7 B). The treatment improved the degree of expression (low to high) in the order of PS-ASO (for 6 weeks) > PS-ASO (for 3 weeks) > Taxotere®.

3.4.5. PS-ASO treatment-mediated improvement of hepatic ultrastructural changes in the carcinogen-treated rats

Scanning electron microscopic images (Fig. 8) have depicted the ultrastructure of the hepatic tissues of the experimental rats. All figures were incorporated with a higher magnification also. Fig. 8 A, B shows the ultrastructure of normal (control) rat liver. Packed arrays of polygonal hepatocytes were seen along in the SEM ultrastructure [37]. The cells had no smooth surface, but predominant sinusoidal spaces were well present. There were prominent canals for central vein, portal veins and arteries present in the tissue. Cells were mostly with uniform sizes. In carcinogen control rat liver (Fig. 8 C, D), the cells were more heterogeneous type, with more tightly packed. Appearance of number of small cells (small round structure, newly grown cells) was seen. Sinusoidal spaces were much less and mostly filled with dead cells and

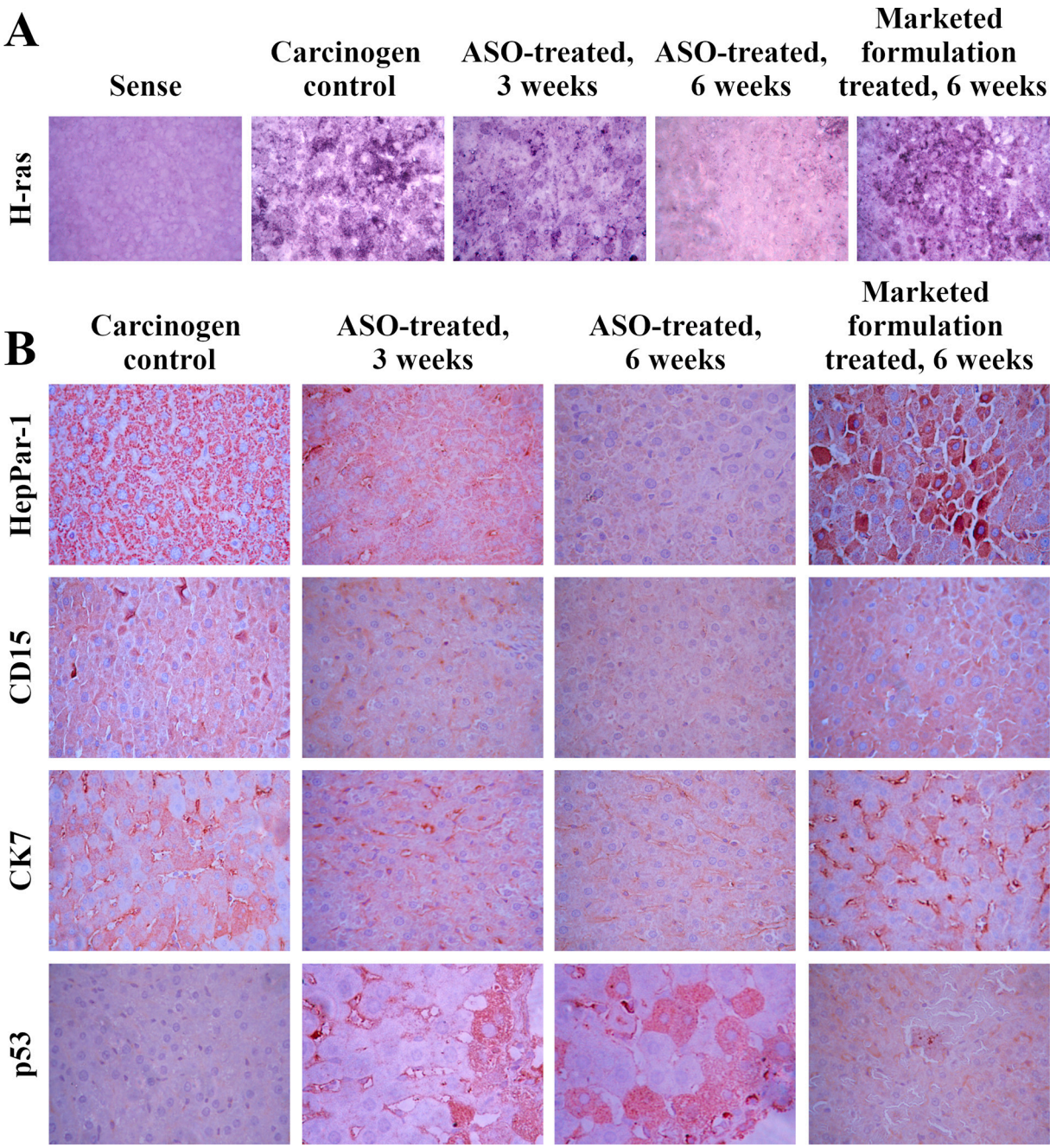


Fig. 7. H-ras gene expression patterns (assessed by in situ hybridization) and various HCC marker protein expressions (determined by immunohistochemical method) in the liver of the experimental rats treated with carcinogen and received the experimental treatments (PS-ASO here represented as ASO in the figure). A. Photographs (40×) show hepatic mutated H-ras gene expression patterns of sense control, carcinogen-induced HCC rats (carcinogen control), and carcinogen-treated HCC rats treated with various experimental treatments. B. Photographs (40×) of hepatic Hep par I, CD-15, CK-7 and p53 protein expression patterns in carcinogen control rats and carcinogen-induced HCC rats received various experimental treatments.

cellular debris. Hepato-portal canals, portal artery and central vein seemed to be narrowed. The cancerous cells showed predominantly increased numbers of microvilli with a larger size on the cell surface. Fig. 8 (E, F) shows hepatic ultrastructure of the carcinogen-treated rat-treated with PS-ASO for 3 weeks. The cells were more round or oval shaped and remained on the tightly packed hepatocytes in areas. The hepatocytes were well connected with the other as in the case of cancerous cells. In some areas, sinusoidal spaces were visible. Fig. 8 (G, H) shows hepatic ultrastructure of the carcinogen-treated rat-treated with PS-ASO for 6 weeks. PS-ASO treatment restored hepatocellular structure more towards normal hepatic architecture. At higher magnifications, normal hepatocytes looked like cuboidal or hexagonal type. The cell surface as in the case of normal hepatocytes was rough (Fig. 8 F and G). Taxotere® treatment did not show a predominant improvement of hepatic ultrastructure in carcinogen-treated rats. Presence of heterogeneous and tightly packed hepatocytes with newly grown small cells, with less organized sinusoidal space structure was observed (Fig. 8 I, J).

Morphometric analysis of the SEM data of the hepatic tissues (Supplementary Table S3) showed that carcinogen control rats had maximum average cellular area, perimeter and circularity compared to normal and other carcinogen treated rats received the experimental treatments. Increased average cellular area and perimeter in carcinogen control rats may be due to the increased number of cells, for enhanced

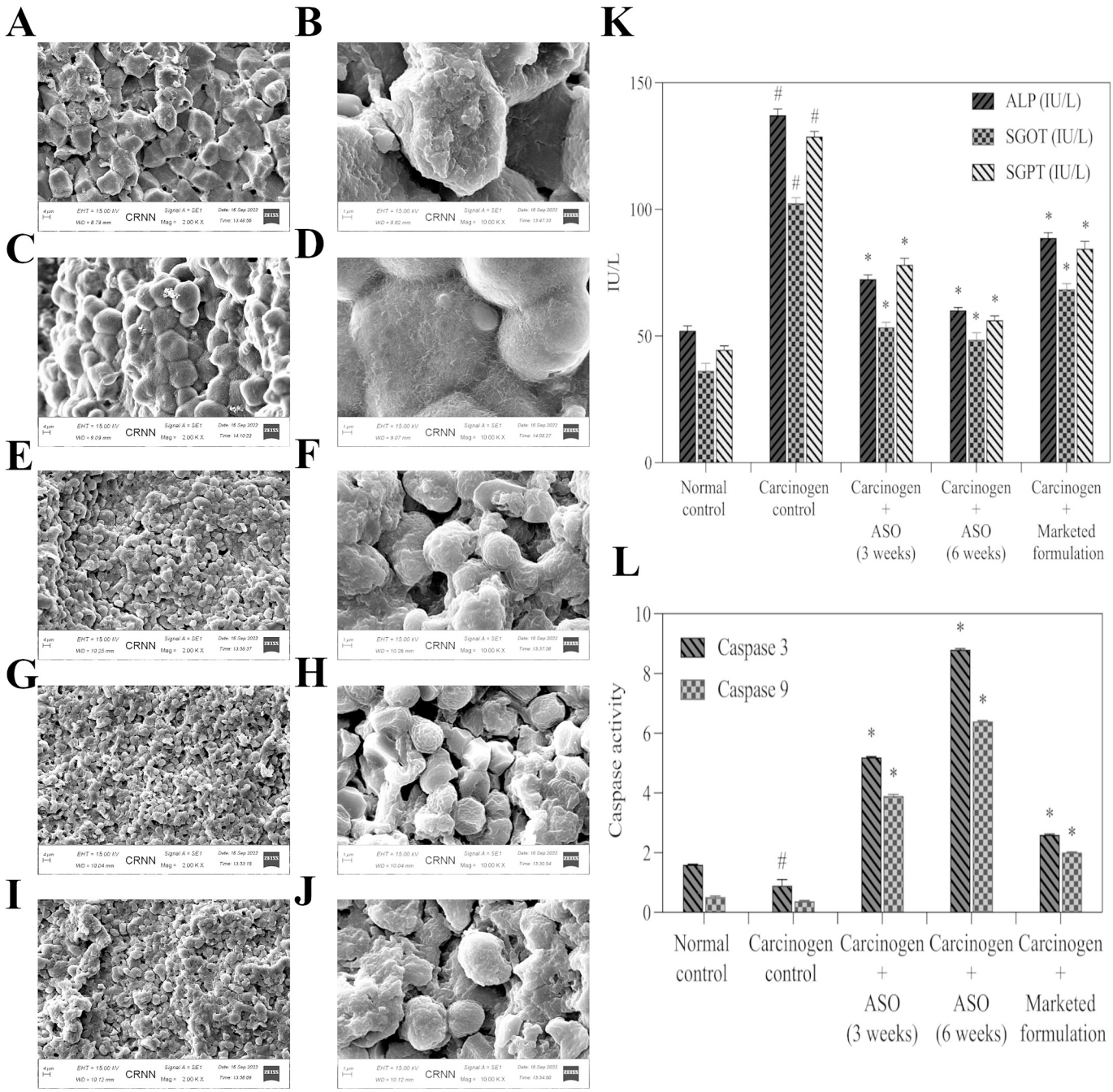


Fig. 8. Scanning electron microscopic images of hepatic tissues, and blood levels of hepatic marker enzymes, and hepatic caspase 3/9 activities of various control and treated experimental rats. PS-ASO is here represented as ASO in the fig. A. Hepatic tissue image of normal (untreated) rats (2000×); B. Portion of the image 8. A, at higher magnification (10,000×); C. Hepatic tissue image of carcinogen-induced HCC rats (2000×); D. Portion of the image 8. C, at higher magnification (10,000×); E. Hepatic tissue image of carcinogen-induced HCC rats treated with PS-ASO for 3 weeks (2000×); F. Portion of the image 8. E, at higher magnification (10,000×); G. Hepatic tissue image of carcinogen-induced HCC rats treated with PS-ASO for 6 weeks (2000×); H. Portion of the image 8. G, at higher magnification (10,000×); I. Hepatic tissue image of carcinogen-induced HCC rats treated with docetaxel formulation for 6 weeks (2000×); J. Portion of the image 8. I, at higher magnification (10,000×); K. ALP, SGOT and SGPT blood levels of various control and treated experimental rats. L. Hepatic caspase 3/9 activities of various control and treated experimental rats. Caspase-3/9 activities are expressed as the fold change in enzyme activity over carcinogen control data obtained from hepatic tissue homogenates. Data (Fig. 8. K and L) show mean±standard deviation (n = 3); *$p < 0.05$ is the statistical level of significance when the data of carcinogen treated rats received various treatments were compared against carcinogen control HCC rats. $^{\#}p < 0.05$ is the statistical level of significance when compared the data of carcinogen control HCC rats with those of normal (untreated) rats. Data were analyzed using one-way ANOVA followed by Student's *t*-test for the data of carcinogen control HCC rats when compared with normal (untreated) control rats, and by Dunnett's *t*-test for the comparison of data of rats received experimental treatments with the data of carcinogen control HCC rats.

cellular proliferation in HCC rats. In 2AAF-induced HCC rats, more oval/round hepatocytes that are usually observed, generate new hepatocytes in rat livers [38], supporting its increased circularity. The average circularity of liver cells of PS-ASO (for three weeks)-treated rats, was between the carcinogen control rats and the other carcinogen-treated rats received PS-ASO/DXT for 6 weeks, suggesting the presence of more round or oval cells in carcinogen-treated rats received PS-ASO treatment for 3 weeks. However, PS-ASO treatment for 6 weeks in carcinogen treated rats improved observed morphometric data more towards normal (untreated control) rats.

3.4.6. PS-ASO treatment reduced ALK, SGPT, and SGOT activities towards normal in the carcinogen treated rats

Aberrant function of the major metabolic organ, the liver, is believed to be a sign of many clinical symptoms. Some crucial biochemical parameters (such as ALK, SGPT, SGOT in blood) that regulate the functions of the organ depict the health of the tissues [4]. Carcinogen control rats showed predominantly elevated levels of ALK, SGPT, and SGOT. PS-ASO/DXT treatment in carcinogen-treated rats reduced the levels of ALK, SGPT, and SGOT (Fig. 8 K). However, PS-ASO-treatment for 6 weeks in carcinogen-treated rats showed the reduction of the enzyme levels more towards normal, suggesting PS-ASO-mediated improvement of hepatic health.

3.4.7. PS-ASO treatment improved the bodyweight of carcinogen treated rats

Bodyweight data were presented on every 5-week basis, except those on the 36th week (provided on a gap of 6 weeks, before scarifying the animals). The carcinogen control rats had the lowest bodyweight on the 36th week (Supplementary Table S4). Carcinogen-treated rats received various experimental treatments had more bodyweight compared to carcinogen control rats from the 20th week. However, DTX treatment in carcinogen treated rats showed less improvement of the bodyweight compared to the other treatments in carcinogen treated rats, suggesting that DTX seemed to be less efficacious and more toxic in the experimental animals in the study. PS-ASO treatment did not show notable reduction of the bodyweight in normal rats compared to the normal untreated rats, suggesting PS-ASO was not toxic to the normal animals at the applied dose. DTX application showed reduction in bodyweight in normal rats, implying its toxic effect in the normal animals.

3.4.8. PS-ASO treatment increased caspase 3 and caspase 9 activities in the experimental rat liver

Elevated caspase 3 and caspase 9 activities are known for induction of apoptosis in cancer cells [39]. We found that caspase 3 and caspase 9 activities in liver were increased predominantly by long-term PS-ASO treatment, followed by PS-ASO treatment for 3 weeks (Fig. 8 L). However, DXT treatment-mediated reduction of those enzyme levels was less than PS-ASO treatments.

4. Discussion

The oligomers were initially characterized for their mass, surface charge, three-dimensional structure using AFM, nucleotide chain-length, TEM and SAEDP analysis. FAM-conjugation to PS-ASO enhanced the molecular mass, zeta potential, and height. PS-ASOs were about 21 nucleotides in length as assessed by agarose gel electrophoresis. The observations suggest that FAM conjugation did not show any unusual alterations of the studied parameters. TEM and SAEDP studies suggest that PS-ASOs had spiral poly-crystalline structure. PS-oligomers with negatively charged backbones were shown to exhibit high plasma protein binding in humans and rodents, causing their sustained action and prolonged stability in blood [31,32,40]. Phosphorothioate backbone modified ASOs were reported to be stable in plasma, for their high nuclease and metabolic stability [39,40]. In vitro hemolytic activity study has demonstrated that PS-ASO administration caused <3 % hemolytic activity between 5 and 100 nM. The safe critical value of hemolytic activity applicable for biomaterials is up to 5 %, as identified by the International Organization for Standardization/Technical Report (ISO/TR 7406.46), suggesting PS-ASO to be used safe for intravenous administration.

Hepatic uptake study suggests that PS-ASOs were distributed rapidly in liver and eventually accumulated with time in the hepatocytes during the period of the investigation. PS-ASOs are known to be metabolically stable in liver, and blood due to the absence of interaction between PS-ASOs, and cytochrome and UDP-glucuronosyltransferase enzymes [41] and their high nuclease stability [42].

Plasma protein binding, and biodistribution, and pharmacokinetic profile of PS-ASOs are mostly driven by the backbone chemistry of PS-ASOs. Several reports showed that PS-ASOs were stable in blood of rats and humans [31,32]. A sharp fall of DTX level in blood was seen from a PS-ASO-equivalent dose of Taxotere® compared to PS-ASO administration. PS-ASO showed higher blood residence time and sustained therapeutic action for its comparatively longer presence in blood. PS-ASO increases its both plasma protein binding and plasma stability, and largely increases tissue distribution and half-life [43]. The study supports the earlier findings that higher PS-ASO half-life in blood, low over all clearance values and higher bioavailability (AUC) of PS-ASOs compared to DTX in the experimental animals. Plasma protein binding of phosphorothioate oligonucleotides limits glomerular filtration and thereby controls urinary excretion of PS-ASOs and enhances tissue distribution, prolonging inhibitory activity [43].

PS-ASO biodistribution was typically with high concentrations in the clearance organs such as liver and kidney followed by lungs. Spleen had considerably low PS-ASO concentration. The PS-ASO concentration was rapidly increased in lungs from 6 h to 8 h. Data showed the potential effect of high and increasing PS-ASO concentrations in liver and lungs in inhibiting tumor incidences, both primary liver cancer (in liver) and metastatic tumor (in lungs). Increasing amount of PS-ASO was also distributed in spleen with time, but with a comparatively much low concentration. Data were supported by the confocal microscopic observations, too.

Liver is the chief metabolic organ, while kidneys are primarily involved in elimination process. Like most other drugs, PS-ASOs are actively metabolized in liver and eliminated through kidneys [44]. Hence, the eventual increase of PS-ASOs in liver and kidneys up to first 8 h (the study period) occurred due to slow PS-ASO release from blood. PS-ASO hinders its glomerular filtration to limit PS-ASO urinary excretion that also causes its kidney accumulation [43]. A report suggests that PS-ASO is mainly distributed in the liver, and kidney followed by spleen [44]. However, spleen had comparatively less PS-ASO accumulation in the present study. Report suggests that following intravenous drug administration, extrahepatic metabolism predominantly occurs in kidneys, and lungs [45]. In our study, an eventual but comparatively rapid increase of PS-ASO level in lungs was observed. In a multiple organ system, drug distribution possesses a significant degree of complexity, based on a variety of factors that include polarity, ionization, size, permeability of drugs, individual genetic variation, disease states and many more [45]. The lungs play a pharmacological active role that often affects intravenously administered drug concentration in blood, and the organ can accrue, metabolize or retain and hinder the release of many drugs or compounds [46]. Because of large lung surface area, and good epithelial permeability with a small aqueous volume, lungs can slowly accumulate small molecules also [46].

Small stable PS-ASOs are rapidly internalized in the hepatocytes using clathrin-mediated endocytosis and endosomal membrane-bound scavenging receptor stabilin [47]. In the early endosomal pathway, Rab5C and EEA1 (early endosomal antigen 1, available in endosomal peripheral membrane with zinc dependent binding motifs) were well-reported proteins for phosphorothioate-ASO trafficking to endosomes in many cancer cell lines, including liver carcinoma HepG2 cells [48,49]. Further, lysobisphosphatidic acid rich late endosomes along

with Rab7A play essential role for PS-ASO escape in the late endocytosis [50], and PS-ASOs thus reach cytoplasm by lysosomal escape [48,49] for functioning by binding with the complementary sequence of targeted site of overexpressed mRNA of the respective gene [51]. It leads to RNase-mediated degradation of mRNA and stops the synthesis of the protein of the gene [9]. PS-ASOs also can cross the nuclear membrane and reach nucleus to block gene through complementary sequences to stop gene expression [51].

Carcinogenic insult causes several genetic and epigenetic alterations [10] that lead to a considerable biochemical and morphological changes in the cells, which ultimately develop preneoplastic lesions [4,10]. There were different preneoplastic lesion types observed in the present experimental model. Many of such preneoplastic lesions eventually develop neoplastic lesions, and dysplastic nodules [4,10]. Finally, HCC arises from the dysplastic/hyperplastic nodules [4,6]. A gross reduction of larger lesions (>3 mm–1 mm) with a presence of the maximum percentage of small preneoplastic lesions (<1 mm) by PS-ASO treatment (for 6 weeks) compared to the other treatments demonstrates that PS-ASO treatment (for 6 weeks) delayed the progress of HCC development maximally in the present experimental rat model. The finding was further supported by the observation of non-appearance of tumor incidences in rats received carcinogen and treated with PS-ASO for 6 weeks. Some carcinogenic rats showed metastatic pulmonary tumors. PS-ASO/DTX treatment inhibited such metastatic tumor growth in lungs.

The histological observation of the tissue sections of liver, kidney, lung and spleen of normal animals received PS-ASO suggests that no predominant histological changes occurred in liver, kidney, lung and spleen. High PS-ASO concentration in normal kidneys did not show any histological evidences of toxicity in the renal tissue, and the finding was supported by the earlier report that chronic administration of PS-ASO in rats did not show any evidences of renal functional abnormalities, but only in some cases low incidence of non-adverse reversible kidney changes [31]. However, Taxotere® treatment for 6 weeks showed some histological alterations in kidneys, lungs and liver, suggesting DTX-mediated toxic manifestation in those tissues.

Elevated expression of *H-ras* gene has been well associated with genesis, progression and maturation of HCC tumors [12,13]. Experimental treatments, more particularly PS-ASO long-term treatment, inhibited the gene expression maximally in the carcinogen treated rats, suggesting that PS-ASO treatment for 6 weeks efficiently controlled *H-ras* expression to inhibit HCC progression. DTX did not predominantly inhibit *H-ras* expression, suggesting it worked without interfering *ras* gene. In fact, DTX arrests mitotic cell division to inhibit HCC [20].

Hep par-1 is a widely accepted immunohistochemical marker for HCC, even for poorly differentiated HCC [25]. In the present study, a very strong Hep par-1 protein expression in carcinogen control rats was supported by the reported findings [25]. Hep par-1, a monoclonal antibody against carbonyl phosphate synthetase of urea cycle, is located in mitochondria and is the most sensitive and specific immunohistochemical marker for HCC and hepatocyte differentiation during carcinogenesis [52]. Inhibitory action on Hep par-1 protein expression by the PS-ASO/Taxotere® treatment and maximum reduction of the protein by PS-ASO long-term (6 weeks) treatment suggest that the treatments controlled HCC progression in rats with a maximum efficacy of the PS-ASO long-term treatment.

CK-7 was reported to be positive in nearly 30 % HCC [27]. CK-7 also expresses strongly in cholangiocarcinoma, cancer of bile ducts that connect liver to gallbladder [26]. PS-ASO long-term treatment showed the maximum reduction CK-7 positive cells and thereby, showed maximum efficacy in controlling CK-7 expression during HCC development.

HCC usually manifests a poor prognosis and differs from other solid tumors as it causes intrahepatic metastasis [53]. Several studies demonstrated a positive correlation between HCC, intrahepatic metastasis, and CD15 expression [28,54]. Strong CD15 expression and development of multiple HCC tumors in the liver of carcinogen control rats thus suggest intrahepatic metastasis. Experimental treatments of PS-ASO (for 3 weeks)/Taxotere® that reduced hepatic CD15 expression in carcinogen treated rats once again support the treatment efficacy in controlling intrahepatic metastasis and HCC progress. However, long-term PS-ASO treatment demonstrated a remarkably high inhibitory efficacy of HCC tumor development.

The most frequently mutated tumor suppressor gene in liver cancer is p53 that regulates several genes associated with cell cycle, cell death, apoptosis, DNA repair, senescence, and cellular metabolism [55]. A very low p53 expression was observed in carcinogen control rats. In HCC, elevated levels of gankyrin, 26S proteasome regulatory protein, p53-specific ubiquitin ligase, and murine double minute 2 decrease p53 level [56,57]. The *miRNA-24* is dysregulated in HCC and the *miRNA* inhibits p53 levels and promotes neoplastic invasion and metastasis [58]. This impaired p53 activity contributes to HCC genesis. However, the experimental treatments in carcinogen treated rats showed the enhanced p53 expression in the order of PS-ASO (for 6 weeks) > PS-ASO (for 3 weeks) > Taxotere®, suggesting the role of the experimental treatments in the inhibition of HCC development by inducing p53 level.

Packed array of mostly hexagonal hepatocytes was seen in the ultrastructure of normal (control) liver. With a clear presence of sinusoidal spaces, portal and arterial canals and short microvilli on cell surface [37,59]. Hepatocyte contains apical (bile canaliculated) membrane and basolateral (sinusoidal) plasma membrane domain, which provided rough surface of the hepatocytes in their ultrastructure [59]. In three dimensional appearances, hepatocytes were polygonal and either contact with sinusoids or neighbouring hepatocytes. A portion of the lateral faces of hepatocytes was modified to form bile canaliculi [59]. In carcinogen control (HCC) livers, hepatocytes were mostly tightly packed with the distinctively increasing appearance of microvilli. Hepatocytes have many microvilli on their surface, which are known to help in the plasma exchange of nutrients and waste [60]. Increased numbers of microvilli are closely associated with the high tumor growth and highly metastatic potential of tumor cells [60]. In fact, high tumor growth was seen in carcinogen control animals, and 3 out of 5 animals showed metastasis in lungs in in the carcinogen control animals. However, upon PS-ASO-treatment, the neoplastic hepatocytes compared to carcinogen control rats appeared much improved towards the normal hepatocytes. Data suggest that PS-ASO treatment definitely improved the ultrastructure of cancerous hepatocytes towards normal.

Bodyweight data suggested that PS-ASO administration for 6 weeks distinctively improved the bodyweight of carcinogen treated rats towards normal (control) rats, suggesting that PS-ASO was efficacious to inhibit HCC. Further, PS-ASO treatment did not notably reduce the bodyweights of normal rats, indicating that it was nontoxic in the normal rats at the given dose.

The experimental treatments improved hepatic health as reflected by the ALK, SGPT, SGOT blood levels. The long-term PS-ASO treatment data suggested for the maximum improvement in the case. Further, activated caspase-9 alters mitochondrial morphology and reactive oxygen species production through Bid into tBid. The enzyme also activates caspase-3 that efficiently executes apoptosis primarily by chromatin condensation and DNA fragmentation [39,61]. The caspase 3 and caspase 9 activities were induced remarkably by long-term PS-ASO treatment, implicating that PS-ASO controlled the pathogenesis of HCC by promoting apoptosis.

5. Conclusions

PS-ASO released in a sustained manner from blood and successfully reached liver of the experimental animals. It has been distributed well in kidney and lung also. It did not show any toxic manifestations in normal animals. PS-ASO therapy (for 6 weeks) showed vividly superior therapeutic potential to commercially available docetaxel in inhibiting chemically induced tumor development in rats. It showed therapeutic

potential by H-ras-mediated apoptotic process, at least by inducing caspase-3/9. PS-ASO also inhibited metastatic lung tumor development. Thus, PS-ASO-mediated gene therapy against the mutated H-ras gene has a great hope in future HCC therapy, and may be implicated to therapy for other cancers. Further studies are warranted.

CRediT authorship contribution statement

Alankar Mukherjee: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Ramkrishna Sen:** Methodology, Investigation, Formal analysis, Data curation. **Ashique Al Hoque:** Methodology, Formal analysis, Data curation. **Tapan Kumar Giri:** Writing – review & editing, Visualization, Supervision, Investigation. **Biswajit Mukherjee:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no potential conflict of interest.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2024.122680.

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