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Potential Anti-cancer Activity of Some Novel Isatin Sulfonamide Derivatives using HepG2 Cancer Cells

A Thesis

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By

Mahmoud Abd Elmoniem Eldeeb

Bachelor Degree in Pharmaceutical Sciences, 2015, From 6th of October University

Pharmacist at the Biochemistry Department, Biotechnology Research Institute National Research Centre

Supervising committee

Dr. Nadia Hamdy Elhefny, Ph.D.
Professor and Head of
Biochemistry Department,
Faculty of Pharmacy,
Ain Shams University

Dr. Mamdouh Moawad Ali, Ph.D.
Professor of Biochemistry and
Vice President for Research Affairs
National Research Centre

Dr. Eman Fouad Sanad, Ph.D.
Lecturer of Biochemistry,
Faculty of Pharmacy,
Ain-Shams University

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والحمد لله رب العالمين.

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AFB1	Aflatoxin B1
AIH	Autoimmune hepatitis
α-SMA	α-smooth muscle actin.
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
CAM	Complementary and alternative medicine
CLL	Chronic lymphocytic leukemia
DAA	Direct acting antiviral
DDT	Dichlorodiphenyltrichloroethane
DM	Diabetes mellitus
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
Dox.	Doxorubicin
DR	Death receptor
EASL	European Association for the Study of Liver
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial mesenchymal transition
FISH	Fluorescent in situ hybridization
GSH	Reduced glutathione
HBV	Hepatitis B virus

HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HepG2	Human liver cancer cell line
HER	Human epidermal receptor
HRP	Horseradish peroxidase
HS	Heparin sulphate
hTERT	Human telomerase reverse transcriptase
LT	Liver transplantation
MDA	Malonaldehyde
MMP	Matrix metalloproteinase
MOMP	Mitochondrial outer membrane permeabilization
NAFLD	Nonalcoholic fatty liver disease
OS	Overall survival
OC	Oral contraceptive
PDT	Photodynamic therapy
PI	Propidium iodide
RFA	Radiofrequency ablation
ROS	Reactive oxygen species
RPE1	Retina pigmented epithelial cell
TCE	Trichloroethylene
TACE	Trans arterial chemoembolization
TNF	Tumor necrosis factor

uPA	Urokinase plasminogen activator.
VEGFR	Vascular endothelial growth factor receptor

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Abstract

The current study investigated the cytotoxic effect of ten newly synthesized isatin sulfonamide derivatives, following molecular hybridization, based on the association principle(s), on hepatocellular carcinoma (HCC) HepG2 and Huh7 cell lines, compared for safety using human normal retina pigmented epithelial (RPE-1) cells. The ten compounds showed variable in vitro cytotoxicity on both HepG2 and Huh7 cells, by using MTT cell viability assay.

Four compounds were highly cytotoxic; however, 3 of them were of highest safety margin on RPE-1 cells *in vitro* as well as *in vivo* acute oral toxicity study (14-days). These later superior three compounds are **(3a)**, **(4b)** and **(4c)** significantly decreased epithelial growth factor receptor (EGFR) level, and confirmed via performing molecular docking study. Having better and selective tyrosine kinase inhibitory potential towards EGFR more than erlotinib.

Compounds 3a, 4b, and 4c inhibited Bcl-2, uPA, heparanase expression in cell lysate from HepG2-treated cells in comparison to doxorubicin-treated HepG2 cells (positive cytotoxic chemotherapeutic control).

In conclusion, new isatin sulfonamides derivative compounds 3a, 4b and 4c showed variable potential on "hallmarks of cancer", significant cytotoxic, anti-cancer activity, and apoptotic anti-angiogenic, anti-invasive, anti-proliferative, and anti-metastatic activities. In summary, compound **"3a"** is highly comparable to Dox. Regarding cell cycle G0-G1 phase % elongation, early and late apoptosis (tested by flow cytometry) and comparable to erlotinib regarding EGFR inhibition.

Therefore, it could be pointed out in the current study that compound **"3a"** is the safest and active synthesized isatin sulfonamide derivative for HCC management.

Key words: Apoptosis, HepG2, Huh7, Isatin sulfonamides, Angiogenesis, Invasion, Metastasis, Cancer hallmarks, Molecular docking, EGFR tyrosine kinase inhibitor.

Hepatocellular carcinoma (HCC) is a terrible public health problem, which ranks the sixth most common cancer all over the world and the fourth most common cause of death from cancer **(Rivara et al. 2016, Forner et al. 2018)**. Egypt is considered the third in Africa and 15th most affected country worldwide **(Craig et al. 2019)**. HCC constitutes more than 70% of all liver tumors among Egyptian patients and about 5% among patients with chronic liver diseases. The primary risk factors for HCC are referred to cirrhosis, Hepatitis C Virus (HCV) and Hepatitis B Virus (HBV) **(Saad et al. 2020).**

Moreover, HCC incidence rate ranks the first cancer between Egyptian males and the second between Egyptian females after breast cancer **(Hussein et al. 2021).** HCC diagnosis may delay for about 3 years from the time of development because of its silent growth **(Sangiovanni and Colombo 2021).**

Isatin is a well-known natural product found in many plants, which is a common scaffold in many drugs, dyes and agrochemicals **(Fayed et al. 2021).** It is found in *Strobilanthes cusia* (Nees) roots and leaves and was first isolated and synthesized by Erdmann and Laurent in 1840 **(Kurahara et al. 1999),** from plants of the *Calanthe discolor, Isatis Couroupita guianesis and tinctoria*. These plants are widely distributed in different regions of China and are registered in folklore medicine. Isatin can be found also in secretion from Caribbean *tumorigenic plant, fungi marine, mollusks* and *Melochia tomentosa*, and the parotid glands of Bufo frogs **(Nath et al. 2021).**

Many isatin derivatives display diverse pharmacological activities, including, anticonvulsant, antitumor, antifungal and antiviral **(Nath et al. 2020).** Because of its unique size and privileged electronic properties, there is considerable interest in the development and pharmacology of isatin derivatives for their diverse activities **(Nath et al. 2020).** Compounds bearing isatin chemical scaffold has been reported to possess diverse activities for the cancer treatment **(Garofalo et al. 2003, Wang et al. 2018).** Therefore, it's believed that anti-tumor drugs which contain isatin scaffold could possess broader spectrum of cytotoxicity to cancer cells. The Michael acceptor (α, β -

unsaturated ketone). Pharmacophore has been considered as a common skeleton that possess by many naturally occurring agents **(Shin et al. 2011, Wang et al. 2018).**

One of most popular strategies to develop new anti-cancer agents is molecular hybrid depend on structural features combination of two different active fragments, which not only enhance the pharmacological outcomes but also decrease the risk of drug-drug interactions **(Fang et al. 2010, Mishra and Singh 2016).**

Most, if not all, human cancers share six mostly acquired capabilities that enable tumor growth. Cancer cells stimulate their own growth and resist inhibitory signals that might stop it. Cancer cells resist apoptosis, promote angiogenesis to supply nutrients to tumor microenvironment, get metastasis that invade local and distant sites **(Yang et al. 2020).**

This work studied the chemo preventive effect of isatin sulfonamide derivatives on HepG2 and Huh7, to choose the most effective compound which inhibits the development of cancer and metastasis.

The most active compounds will be subjected to the biochemical studies including:

1. MTT assay,
2. Apoptosis/necrosis analysis using Annexin V/PI double staining,
3. Cancer growth to be detected by uPA levels in cell lysate,
4. Cancer metastasis indicator by heparanase activity in the cell lysate,
5. Angiogenesis quantification of epidermal growth factor receptor (EGFR) levels in the cell lysate,
6. Apoptosis via BcL-2,
7. Assessment of lipid peroxidation by measuring the level of malonaldehyde (MDA).
8. Assessment of antioxidant activity by measuring the level of reduced glutathione (GSH),
9. Molecular Docking study using Molecular Operating Environmental (MOE) software (Version10.2008, Chemical Computing Inc., Montreal, Quebec, Canada).

This study characterized the newly synthesized isatin sulfonamide derivatives. Isatin was used as a starting material for synthesis of 3-hydroxy-3-substituted-2-oxindoles through the reaction of isatin derivatives with acetophenone derivatives. Together with, preparing 3-phenacylidene-2-indolinone derivatives and finally synthesis of some arylidene derivatives to be used as a new possible drug for treatment of HCC at *in vitro* level using HepG2 and Huh7 cell lines.

The most active and safest synthesized compound(s) will be evaluated for their possible anti-cancer potential, apoptotic, cellular cytotoxicity, angiogenesis arrest and metastasis inhibition as well as oxidative stress induction, against two hepatic cancer cell line model(s), followed by investigating cell death mechanism underlying this activity, with further inhibitory mechanism confirmation using molecular docking (MD) study, together with ensuring safety tested using normal human retinal cell line, together with performing an *in vivo* acute oral toxicity study. To elucidate the chemo-preventive effect of isatin sulfonamide derivatives on hepatocellular carcinoma cell lines the next objectives were made:

- **Objectives:**

1. To test if isatin sulfonamides inhibit, reverse or restrict the development of cancer.
2. To test the possible inhibitory effect on cancer metastasis, apoptosis, and angiogenesis.
3. To Test the effect of these new compounds on cell cycle.
4. To Test these cancer modulators safety both *in-vitro* and *in-vivo*.
5. Molecular docking study.

1. HCC epidemiology

HCC is a serious disease which is considered the sixth most common cancer all over the world **(Inchingolo et al. 2021).** The fourth commonly cause of cancer death globally and the first cause of cancer death in Egypt, Egypt is the 3rd in Africa and 15th most affected country worldwide **(Craig et al. 2019).**

HCC is a highly public health concern, representing more than 85% of liver cancers worldwide. The diagnosis of HCC may be delayed due to the absence of sensitive, specific and early biomarkers **(Abdel-Hamid et al. 2018).**

Asia and Africa have the highest incidence rates of HCC. In multiethnic countries, racial and ethnic minorities experience disparities in HCC incidence as well as mortality, representing an essential area for improvement in terms of healthcare inequity **(Konyn et al. 2021).**

Primary prevention of major public health threats by 2030 is one of the World Health Organization (WHO) strategies. Sustainable development goals include eradication of viral hepatitis, decreasing the harmful consumption of tobacco, alcohol and decreasing diabetes and obesity **(WHO (Organization 2016)**.

2. HCC risk factors (table 1)

HCC is considered the most frequent tumor, associated with high mortality rate Pathogenesis of HCC contributes to several risk factors **(Ghouri et al. 2017)**.

HCC would occur in the absence of cirrhosis, pre-existing cirrhosis is an important indication for hepatocarcinogenesis because of the high rate of co-existing cirrhosis in HCC patients **(Alqahtani and Colombo 2020)**. Other chronic liver diseases, can lead to HCC with a proportion less than 5% to 10% globally such as genetic or metabolic liver diseases and chronic biliary disease, these can cause cirrhosis and induce the development of HCC **(Yang and Roberts 2010)**.

Table 1: HCC risk factors

I-Environmental related risk factors		II-Host related risk factors
1.Infectious	**2. Non-infectious**	**a) Oral contraceptives**
		b) Pesticides
a) HBV	**a) Alcohol abuse**	**c) Obesity**
b) HCV	**b) Smoking tobacco**	**d) Diabetes Mellitus**
		e) NAFLD
		f) Iron overload
		g) Autoimmune Hepatitis

2.1 Environmental-related risk factors

2.1.1 Infectious viral hepatitis B and C

Hepatitis is an inflammatory condition that can develop HCC **(Selim et al. 2019)**. HCC has several etiological risk factors like HBV and HCV, which have a strong contribution in development and increasing the risk of HCC by two to six folds. About 80%–90% of HCC cases are due to cirrhosis **(Ghouri et al. 2017).**

2.1.2 Non-infectious

a) Alcohol abuse

In USA and Europe, the second most common risk factor for HCC is alcoholic cirrhosis **(Park et al. 2015).** The elevated risk of developing HCC in patients with cirrhosis from chronic viral hepatitis is higher than in patients with alcohol-related cirrhosis **(Demirtas and Brunetto 2021).**

b) Smoking tobacco

The effect of tobacco which is metabolized in liver is biologically acceptable in the development of HCC because of the carcinogenic effect of several ingredients in tobacco**.** In Egypt, patients used to smoke for more than 20 years