CHAPTER-4

Synthesis of hydrazono-isatin and N-methyl-isatin derivatives
4.1. Theoretical

Isatin is an indole derivative i.e. 1H-indole-2,3-dione (120). In 1841, isatin was obtained as a product from the oxidation of indigo dye by nitric acid and chromic acid by Erdmann and Laurent for the first time.

For the synthesis of large number of heterocyclic compounds such as indole derivatives, quinoline derivatives and raw material for drug synthesis, isatin nucleus can be used. Because of this mentioned fact isatins are synthetically versatile nuclei.

Sammaiah et al. reported the synthesis of 2-aminobenzoic acid(2-oxo-1,2-dihydro-indol-3-ylidene)hydrazides (123) by the reaction of suitable isatin (121) and 2-aminobenzoic acid hydrazide (122) in alcohol containing few drops of acetic acid.
Spiro thiazolidinone-isatin conjugates (128) were prepared by Kaminskyy et al.\cite{4} by the reaction of isatin, appropriate amine (126), acetic acid and thioglycolic acid (124) under reflux conditions. Also, microwave assisted organic synthesis approach was used for the synthesis of target compounds (127) by reacting thioglycolic acid (124), isatin (120) and amine derivative (125).

\[ \text{OH} + \text{SH} \xrightarrow{\text{microwave}} \text{O} \]

\[ \text{~O} \]

\[ \text{~A} \]

\[ \text{H}_2\text{N} \]

\[ \text{O} \text{ydrazono-isatin and N-methylisatin derivatives} \]

\[ \text{Spiro thiazolidinone-isatin conjugates (128) were prepared by Kaminskyy et al.}\cite{4} \text{by the reaction of isatin, appropriate amine (126), acetic acid and thioglycolic acid (124) under reflux conditions. Also, microwave assisted organic synthesis approach was used for the synthesis of target compounds (127) by reacting thioglycolic acid (124), isatin (120) and amine derivative (125).} \]

\[ \text{N}^4\text{-Aryl substituted-5-trifluoromethoxy isatin-3-thiosemicarbazones (131) were prepared by Pervez et al.}\cite{5} \text{by the reaction of trifluoromethoxy isatin (129) with different aryl thiosemicarbazides (130) in aqueous ethanol containing few drops of acetic acid.} \]

\[ \text{[87]} \]
The synthesis of 3-substituted isatin derivatives (134) were described by Nagarajan et al. by the reaction of substituted isatin (132) with amino derivatives of lamotrigine (133).
Bis-Schiff bases isatin and its derivatives (137 and 139) were prepared by Jarrahpour et al.\textsuperscript{7} by the reaction of isatin, 5-fluoroisatin and benzyl isatin (120 and 135) with aromatic diamines (136 and 138) in the presence of catalytic amount of glacial acetic acid in ethanol.

\[
\text{(135)} \quad \text{(136)} \quad \text{EtOH, reflux} \quad \text{(137)}
\]

\(X: \text{CH}_2, \text{O, CO}; \ Y: \text{H, Cl}; \ Z=\text{H, Et}; \ W: \text{H, Et}; \ R^1: \text{H, Bn}; \ R^2: \text{H, F}\)
Panda et al. reported the green chemistry approach for the synthesis of Schiff bases of isatin derivatives (141) by the condensation of keto group of isatin (120) with different aromatic primary amines (140) under microwave heating method.

In most of the developed countries after cardiovascular diseases, cancer represents one of the leading causes of mortality. The mainstay in the treatment of malignancies is the use of chemical agents to destroy cancer cells i.e. chemotherapy. Chemotherapy is used to treat widespread or metastatic cancers whereas radiation therapies and surgery are limited to treating cancers that are restricted to specific areas. Nowadays, significant research work is dedicated to introduce more affective anticancer agent
while minimizing their toxic side effects, although many antineoplastic agents have been successfully identified and used in cancer treatments. Several drug delivery approaches have been modified to intensify the effectiveness of anticancer agents due to invariant advancements achieved in the field of chemistry, nanotechnology, soft matter science and in the understanding of biological mechanisms of cancer. Most of the drugs works by interfering with the cellular functions by microtubule assembly inhibition, by intercalation of DNA with groove binding, by proteins and nucleic acids alkylation, by topoisomerase II inhibition and by microtubules polymerization stabilization or by biosynthesis prevention or by normal cellular metabolites utilization. Microtubules are a sensitive target for the development of anticancer drugs because drugs that bind to tubulin act by interfering with the mitosis of cells during the M-phase resulting in mitotic arrest and ultimately leading to apoptosis. In 2006, for the treatment of gastro intestinal stromal tumors and advanced renal cell carcinoma, SU11248 (Sutent), a 5-fluoro-3-substituted-2-oxoindole IUPAC name: N-(2-diethylaminoethyl)-5-[(Z)-(5-fluoro-2-oxo-1H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrolo-3-carboxamide, (142) was approved by the US FDA.

Some halogenated derivatives of isatin were found to exhibit antileukemic activity against P388 lymphocytic leukemia in mice and 5-bromo-3-o-nitrophenyl isatin hydrazone was found to be active intramuscularly against Walker carcinoma-256. In multicellular organisms, there are two patterns of cell death: necrosis and apoptosis. Apoptosis also known as programmed cell death, is a type of cell death that occurs during various pathological situations in multicellular organisms and help to cause replacement of cells, damaged cells removal under normal conditions and
remodeling of tissue. In normal cellular processes including maintenance of the immune system, fetal development and tissue homeostasis, apoptosis plays a significant role. In pathological conditions such as allograft rejection, cardiomyopathy, neurodegeneration, ischemia-reperfusion injury and sepsis, abnormal apoptosis has been found. These all are cited in the literature. Apoptosis is characterized by well known cytological and molecular events which include a change in the refractive index of cells, cytoplasmic blebbing, nuclear shrinkage and condensation of chromatin, shape irregularity of cells, retraction of processes, internucleosomal DNA fragmentation, cleavage of poly(ADP-ribose)polymerase (PARP) and apoptotic bodies formation. In pharmaceutical industry, the synthesis of novel drugs that can stop the process of apoptosis has been one of the most important and significant area of research.

On the other hand literature survey also reveals that isatin structural motif can be found in a broad range of natural and synthetically derived biologically and pharmacologically active compounds. Isatin derivatives have been also found to possess antimicrobial, antiviral, anticonvulsant, antitubercular, antioxidant, anti HIV, anthelmintic, antiplasmodial, antiinflammatory and analgesic activities.
4.2. Synthesis and Studies Involving Apoptosis Induced by Novel Isatin/N-Methyl Isatin Derivatives of Fatty Acid Hydrazones*

Recently, some of the hydrazone derivatives have been reported as antitubercular agents whose mechanism of action was same as already known drug: isoniazid derivatives. Nowadays, in the treatment of neuropsychological disorders such as depression and schizophrenia, fatty acid derivatives are used. Some of the fatty acid derivatives were reported as potent antitumor agents which play an important role in tumor growth progression. Also, the heterocyclic derivatives of saturated fatty acid i.e. stearic acid heterocyclic derivatives have been found to possess antidepressant and antimicrobial activities. Few years back, in our laboratory number of novel series of heterocyclic analogs of selected fatty acids (saturated, hydroxy-unsaturated, non hydroxy-unsaturated) have been synthesized and were found to be biologically active. It has been described in reviews that isatin derivatives possessing important biological activities as well as the isatin analogs were also found to possess antimalarial, antitubercular, antimicrobial, etc. These aforementioned facts about the heterocyclic analogs of fatty acids and isatin derivatives prompted us to design and synthesize a series of novel compounds based on heterocyclic indole scaffold nucleus to assess their cytotoxicity profile. In this chapter, novel hydrazono-isatin analogs of fatty acids were synthesized by using fatty acid hydrazides as starting material for the first time. On the molecular design level, different fatty acid hydrazides were condensed at position 3 of isatin/N-methyl isatin nucleus and impact of this performed manipulation was studied from the obtained cytotoxicity assessment and apoptosis induction using MTT assay, propidium iodide (PI) staining, PARP cleavage and fluorescence-activated cell sorting (FACS) assay studies of the synthesized compounds against different human cancer cell lines.

*Research paper entitled “Synthesis and Studies Involving Apoptosis Induced by Novel Isatin/N-Methyl Isatin Derivatives of Fatty Acid Hydrazones” is communicated. (Aiman Ahmad, Varshney, H., Rauf, A. Arab. J. Chem.)
4.3. Results and discussion

4.3.1. Chemistry

The synthesis of the hitherto unreported long chain title compounds and anticancer properties is described in this chapter. The long chain substituted hydrazono-isatin derivatives (144-148) were achieved in excellent yield using condensation reaction (dehydration) with isatin and fatty acid hydrazides (21-25) in ethanol as solvent containing catalytic amount of glacial acetic acid. Similarly, long chain substituted hydrazono-N-methyl isatin derivatives (149-153) were achieved in excellent yield in the same way as discussed above for N-methyl isatin. The schematic representation of the synthetic route of these novel title compounds is shown in Scheme 6. The structures of all the newly synthesized compounds were elucidated by IR, $^1$H NMR, $^{13}$C NMR and mass spectral studies.

For instance, IR absorption bands at 3398 (hetero N-H) and 3063 (amide N-H) were obtained for 3-(undec-10'-enoyl hydrazono) isatin (145). Also, bands at 1690 and 1657 cm$^{-1}$ were observed for C=O stretching vibrations and a band at 1593 cm$^{-1}$ for C=N was observed. $^1$H NMR spectra showed singlet for two N-H protons at $\delta$11.78 and at 9.65. The spectrum also shows a multiplet for four aromatic protons at $\delta$7.62-7.00. For a methine proton at C-10, a triplet of doublet of doublet was observed at $\delta$5.80. C-11 methylene protons designated as $H_z$ and $H_e$ which gave two values of $\delta$ after coupling with adjacent C-10 methine protons. Two doublets of doublet were obtained for $H_z$ and $H_e$ at $\delta$5.00 and 4.92, respectively. $^{13}$C NMR also confirmed the structure of (145). The characteristic signals for C=O, C=N and aromatic carbons were observed at $\delta$176.8, 171.2, 163.2, 142.8 and 141.4-131.7, respectively. The structure of compound (145) was further supported by the mass spectrum which showed a molecular ion peak at m/z 349.368 consistent with the molecular formula. For 3-(undec-10'-enoyl hydrazono)-N-methyl isatin, (150), in addition to the peaks of compound (145) as discussed above, a singlet was observed at $\delta$3.23 for methyl protons which is substituted at nitrogen of indole moiety. A broad singlet which was the evidence of N-H proton of indole in compounds (144-148) was disappeared in the $^1$H NMR spectra of compounds (149-153).
Hydrazono-isatin and N-methyl isatin derivatives

\[
\text{R-CONHNH}_2 \quad (21-25) \quad + \quad \text{EtOH, Cat. AcOH, } \Delta \\
\rightarrow \quad \text{NNHCOR} \quad \text{R': H, CH}_3 \\
\text{(120, 143)}
\]

Scheme 6: Synthesis of novel long chain hydrazono-isatin and N-methyl isatin derivatives

<table>
<thead>
<tr>
<th>Compound Codes</th>
<th>R</th>
</tr>
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<tbody>
<tr>
<td>21, 144, 149</td>
<td>[11CH2]</td>
</tr>
<tr>
<td>22, 145, 150</td>
<td>[7CH2]</td>
</tr>
<tr>
<td>23, 146, 151</td>
<td>[5CH2]</td>
</tr>
<tr>
<td>24, 147, 152</td>
<td>[5CH2]</td>
</tr>
<tr>
<td>25, 148, 153</td>
<td>[5CH2]</td>
</tr>
</tbody>
</table>

[95]
4.3.2. Biology

The synthesized compounds were tested for *in vitro* cytotoxicity by MTT assay against HeLa (cervical carcinoma) cells. Compounds (148 and 153) were found to show best results for cytotoxicity against HeLa cells, so these two compounds were further tested for *in vitro* cytotoxicity against MCF 7 (breast carcinoma), Hep G2 (hepatocarcinoma) and U2OS (osteosarcoma) cell lines. In addition, the induction of apoptosis by these two compounds is evidenced by the PARP cleavage studies through western blotting technique, FACS assay and PI staining results\textsuperscript{31-34}.

4.3.2.1. *In vitro* cytotoxicity

*In vitro* cytotoxicity of the newly synthesized compounds (144-146, 148-151 and 153) was determined by MTT assay against HeLa cells. The general description of MTT assay and cytotoxicity is given in chapter 3 (page number 65, 66), in section 3.3.2.1. Experiment was performed in a triplicate. For each of the tested compounds IC\textsubscript{50} was calculated and the results are summarized in Table 9. Further, cytotoxicity of two selected compounds (148 and 153) was carried out against Hep G2, MCF 7 and U2OS cells by MTT assay. Selection of these compounds was based on low IC\textsubscript{50} value among all the tested compounds against HeLa cells.
Table 9: Anticancer data (IC$_{50}$ values in $\mu$g/mL) of the synthesized compounds against four different human cancer cell lines. Data expressed here is mean±standard deviation of three independent experiments.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Compound Codes</th>
<th>HeLa</th>
<th>MCF 7</th>
<th>U2OS</th>
<th>Hep G2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>144</td>
<td>&gt;50</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>2</td>
<td>145</td>
<td>19.07±1.5</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>3</td>
<td>146</td>
<td>&gt;50</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>4</td>
<td>148</td>
<td>16.19±1.7</td>
<td>15.30±2.5</td>
<td>21.80±2.4</td>
<td>19.63±1.8</td>
</tr>
<tr>
<td>5</td>
<td>149</td>
<td>&gt;50</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>6</td>
<td>150</td>
<td>32.75±0.9</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>7</td>
<td>151</td>
<td>&gt;50</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>8</td>
<td>153</td>
<td>12.21±1.6</td>
<td>18.14±2.1</td>
<td>17.27±3.1</td>
<td>10.64±2.8</td>
</tr>
</tbody>
</table>

N.T.: Not Tested

4.3.2.2. Propidium iodide (PI) nuclear staining

Fluorescence microscopic investigations showed that test compounds induced cell death in Hela cell lines by apoptosis (Figure 3). Phenoitically, apoptosis is identified by cell shrinkage, chromatin compaction, blebbing of plasma membrane, fragmentation of DNA and cell collapse into small intact fragments (apoptotic bodies). Figure 3 shows the morphology of Hela cells treated with compounds (148 and 153) and stained with PI. The fluorescent images showed structure and shape of control cells, untreated cells possessed intact nuclei; cells treated with compound (148 and 153) at different concentrations caused the formation of clear apoptotic bodies and membrane blebbing which leads to cell death.
Hydrazono-isatin and N-methyl-isatin derivatives

<table>
<thead>
<tr>
<th>(a) Control</th>
<th>(b) Positive Control</th>
<th>(c) 148 10µg/mL</th>
</tr>
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<tbody>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>(d) 148 20µg/mL</th>
<th>(e) 153 10µg/mL</th>
<th>(f) 153 20µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</table>

Figure 3: Fluorescence microscopic study of test compounds on Hela cell line stained with 25 µg/mL propidium iodide. (a) Un-stimulated (b) 5 µg/mL Doxorubicin treated (c) 10 µg/mL (148) treated (d) 20 µg/mL (148) treated (e) 10 µg/mL (153) treated (f) 20 µg/mL (153) treated

4.3.2.3. Flow cytometry analysis of cell viability by PI uptake

In apoptosis, membrane bound apoptotic bodies are formed by articulated breakdown of the cells. To determine the live and dead cell caused by compound (148 and 153) in Hela cells, the FACS assay was done using PI staining. PI can be used to stain dead cells so that they may be excluded from analysis in standard live cell surface staining protocols. These dyes cannot pass through intact cell membranes, but may freely enter cells with compromised cell membranes. Upon entering dead cells, PI will intercalate into double-stranded DNA or double-stranded RNA in a stoichiometric manner. As shown in Figure 4, after 72 hours treatment with compounds (148 and 153) at 10 and 20 µg/mL, the number of apoptotic cells was increased in concentration dependent manner.
Figure 4: Flow cytometry analysis using PI staining showing live and dead cell percentage. (a) untreated (b) 5 µg/mL doxorubicin treated, (c) 10 µg/mL (148) treated, (d) 20 µg/mL (148) treated, (e) 10 µg/mL (153) treated, (f) 20 µg/mL (153) treated

4.3.2.4. Apoptosis induction via PARP cleavage

Apoptosis involves the caspases activation and is also implicated in physiological and pathological processes. PARP cleavage has been served as one of the hallmark of apoptosis and caspase activation. To confirm the apoptosis induction by compounds (148 and 153), the expression of cleaved PARP was investigated by western blotting technique. As shown in Figure 5, the cleaved PARP was significantly increased after treatment of Hela cells with compounds (148 and 153) at concentrations of 10 and 20 µg/mL after 72 hours. These results together indicate that compounds (148 and
5-fluorodrazino-isatin and \( N \)-methyl isatin derivatives caused HeLa cancer cell death via apoptosis by increasing the level of cleaved PARP.

![Western blot analysis of PARP cleavage](image)

Figure 5: Western blot analysis of PARP cleavage. Both full length and cleaved forms (p89) of PARP are shown.

4.3.3. Structure-activity relationship (SAR) studies

The synthesized compounds, (144-146, 148-151 and 153) were tested for cytotoxicity assay against HeLa cells. Previously some fatty acid derivatives were also tested for anticancer activities and they were found to possess anticancer properties. Compounds (147 and 152) have not been tested for cytotoxicity because these are the structural-position isomers of compounds (148 and 153), respectively; they differ only in position of double bond and hydroxyl group with each other and it is already known that position isomers show same chemical properties and slightly different physical properties. From cytotoxicity assay results it has been found that, compounds (144, 146, 149 and 151) were not showing significant cytotoxicity against HeLa cells, except compounds (145, 148, 150 and 153) which show cytotoxicity against HeLa cells. The difference in biological activities of all the tested compounds may be related to their structures. So by analyzing the IC50 values it may be said that isatin/\( N \)-methyl isatin derivatives substituted with saturated long fatty acid hydrazone chain (C16) (144 and 149) were not showing significant cytotoxicity. On the other hand, presence of terminal double bond in the fatty acid hydrazone chain (C11) substituted to isatin/\( N \)-methyl isatin derivatives (145 and 150) lead to show some cytotoxicity against HeLa cells. Further, the cytotoxicity assay of compounds (146 and 151) show
inconsiderable results and it may be said that as the number of carbon atoms of fatty acid hydrazone substituted to isatin/N-methyl isatin derivatives (146 and 151) increases (C18) and the terminal double bond changes to internal double bond lead to insignificant cytotoxicity against HeLa cells. Cytotoxicity assay results showed that introduction of hydroxyl group to internal alkenyl chain (C18) substituted to isatin/N-methyl isatin derivatives (148 and 153) leads to remarkable increase in cytotoxicity (having low IC₅₀ value). Among compounds (148 and 153), compound (153) shows better cytotoxicity against cancerous cell lines and we have also found that compound (153) was the better apoptosis inducing agent than compound (148), may be due to presence of N-methyl substituted isatin.

4.3.4. Conclusion

In summary, two novel series of isatin and N-methyl isatin derivatives of fatty acid hydrazones (144-153) were successfully synthesized and characterized in this chapter. Additionally, these compounds were evaluated for in vitro cytotoxicity against four different human cancer cell lines by MTT assay. Further, the apoptosis induction by the compounds was also proved by PARP cleavage studies done by western blotting technique, FACS assay and PI staining results. From the current investigation, structural-activity relationship of these compounds suggests that the nature of fatty acid hydrazone chain (saturated/unsaturated; terminal/internal double bond) and presence of hydroxyl group on fatty acid hydrazone chain may be responsible for difference in biological activities of the synthesized compounds (144-153). Compounds (148 and 153) with hydroxyl group substituted on fatty acid hydrazone chain were found to be most potent cytotoxic compounds and apoptosis inducing agents. Compound (153) was found to be better cytotoxic compound and potentially better apoptosis inducing agent compared to (148) may be due to substitution of N-atom of isatin.
4.4. Experimental

4.4.1. Chemistry

Physical and Spectroscopic Measurements

The source of olefinic and hydroxy-olefinic fatty acids, instrumental details of IR, NMR and mass spectrometry are already detailed in experimental section of chapter 1 (page number 19). Isatin and N-methyl isatin were purchased from Sigma Aldrich. All the reactions were monitored by thin layer chromatography (TLC). The developing solvent used were the mixtures of petrol-acetone-acetic acid (90:10:1; v/v). Anhydrous conditions were achieved by drying flasks and other equipments in the oven. Reagents were of commercial grade and used without further purification. When needed, solvents were dried and distilled before use.

General procedure for the synthesis of long chain alkyl/alkenyl/hydroxyalkenyl hydrazides, (21-25)

The long chain alkyl/alkenyl/hydroxyalkenyl hydrazides (21-25) were synthesized by following the method and these selected fatty acid hydrazides were used as the starting material for the synthesis of long chain alkanoyl/alkenoyl/hydroxyalkenoyl hydrazono-isatin/N-methyl isatin derivatives (144-153).

Synthesis of 3-substituted long chain alkanoyl/alkenoyl/hydroxyalkenoyl hydrazono-isatin/N-methyl isatin derivatives, (144-153)

A mixture of long chain alkyl/alkenyl/hydroxyalkenyl hydrazides (21-25) (0.01 mole) and isatin/N-methyl isatin (0.01 mole) in ethanol (50 mL) containing 5-8 drops of glacial acetic acid was refluxed on oil bath for 6-8 hours and left over night at room temperature. Excess solvent was evaporated and the reaction mixture was poured into crushed ice after consumption of all reactants. The product was washed with cold ethanol several times. Products were purified by column chromatography. All the
Hydrazono-isatin and N-methyl isatin derivatives

newly synthesized compounds (144-153) were characterized on the basis of their spectral data (IR, $^1$H NMR, $^{13}$C NMR and mass spectra).

The spectroscopic and analytical data for the synthesized compounds (144-153) are presented below:

3-(Hexadecanoyl hydrazono) isatin, (144)

Yellow powder; yield: 90%; m.p.: 91-94°C

$IR$ ($KBr$, cm$^{-1}$): 3409 (hetero N-H stretching), 3066 (amide N-H stretching), 2919 (C-H stretching), 1688, 1660 (C=O stretching), 1588 (C=N stretching).

$^1$H NMR ($CDCl_3$, $\delta_H$): 12.98 (1H, s, NH indole), 9.38 (1H, s, NH), 7.36-7.10 (4H, m, ArH), 2.83 (2H, t, $J = 7.61$ Hz, CH$_2$CO), 1.74 (2H, m, CH$_2$CH$_2$CO), 1.25 (24H, br.s, (CH$_2$)$_{12}$ chain), 0.87 (3H, dist.t, CH$_3$).

$^{13}$C NMR ($CDCl_3$, $\delta_C$): 177.1, 170.4, 163.2, 141.9, 139.1, 133.2, 131.7, 131.2, 129.0, 33.8, 32.0, 31.9, 31.6, 29.6, 29.5, 29.3, 29.1, 28.9, 25.2, 24.5, 22.6, 21.9, 21.5, 14.1.


3-(Undec-10'-enoyl hydrazono) isatin, (145)

Yellow powder; yield: 85%; m.p.: 88-89°C

$IR$ ($KBr$, cm$^{-1}$): 3398 (hetero N-H stretching), 3063 (amide N-H stretching), 2920 (C-H stretching), 1690, 1657 (C=O stretching), 1593 (C=N stretching).

$^1$H NMR ($CDCl_3$, $\delta_H$): 11.78 (1H, s, NH indole), 9.65 (1H, s, NH), 7.62-7.00 (4H, m, ArH), 5.80 (1H, tdd, $J_{H-CH} = 6.6$ Hz, $J_{H-N} = 10.1$ Hz, $J_{H-N} = 17.2$ Hz, CH$_2=CH$), 5.00 (1H, dd, $J_{H-C} = 10.1$ Hz, $J_{H-C} = 1.4$ Hz, H$_2$C=CH), 4.92 (1H, dd, $J_{H-C} = 17.2$ Hz, $J_{H-C} = 1.4$ Hz, H$_2$C=CH), 2.79 (2H, t, $J = 7.60$ Hz, CH$_2$CO), 2.03 (2H, m, CH$_2=CH$-CH$_2$), 1.72 (2H, m, CH$_2$CH$_2$CO), 1.31 (10H, br.s, CH$_3$(CH$_2$)$_2$ chain).
Hydrazono-isatin and N-methyl isatin derivatives

\[^{13}C\text{ NMR (CDCl}_3, \delta_C): 176.8, 171.2, 163.2, 142.8, 141.4, 139.7, 137.3, 133.2, 131.7, 123.3, 123.0, 33.9, 33.1, 32.0, 31.7, 29.5, 25.1, 24.5, 22.6.\]

\[\text{MS (ESI): m/z = 349.368 found [M+Na]^+}, \text{ calculated [M+Na]^+ = 350.367.}\]

3-[(9'Z)-Octadec-9'-enoyl hydrazono] isatin, (146)

Yellow powder; yield: 80%; m.p.: 95-96°C

\[\text{IR (KBr, cm}^{-1}): 3372 \text{ (hetero N-H stretching), 3044 \text{ (amide N-H stretching), 2920 \text{ (C-H stretching), 1682, 1669 \text{ (C=O stretching), 1599 \text{ (C=N stretching).}}\]

\[^{1}H\text{ NMR (CDCl}_3, \delta_H): 12.10 \text{ (1H, s, NH indole), 9.65 \text{ (1H, s, NH), 7.56-6.90 \text{ (4H, m, ArH).}}\]

\[5.30 \text{ (2H, m, CH=CH), 2.76 \text{ (2H, t, J = 7.60 Hz, \text{CH}_2\text{CO), 1.99 \text{ (4H, m, CH}_2\text{CH=CHCH}_2, 1.64 \text{ (2H, m, CH}_2\text{CH}_2\text{CO), 1.27 \text{ (20H, br.s, (CH}_2\text{)}_{10} \text{ chain), 0.86 \text{ (3H, dist.t, CH}_3).}}\]

\[^{13}C\text{ NMR (CDCl}_3, \delta_C): 175.8, 174.9, 161.5, 143.0, 142.9, 135.0, 131.4, 130.9, 129.2, 125.3, 123.6, 35.6, 32.0, 31.8, 31.2, 31.0, 30.9, 30.7, 30.2, 29.6, 29.5, 29.3, 29.0, 28.9, 27.3, 14.2.\]

\[\text{MS (ESI): m/z = 449.740 found [M+Na]^+}, \text{ calculated [M+Na]^+ = 448.528.}\]

3-[(9'Z, 12'R)-12'-Hydroxyoctadec-9'-enoyl hydrazono] isatin, (147)

Yellow solid, yield: 75%; m.p.: 85-86°C

\[\text{IR (KBr, cm}^{-1}): 3410 \text{ (O-H stretching), 3368 \text{ (hetero N-H stretching), 3105 \text{ (amide N-H stretching), 2918 \text{ (C-H stretching), 1691, 1654 \text{ (C=O stretching), 1585 \text{ (C=N stretching).}}\]

\[^{1}H\text{ NMR (CDCl}_3, \delta_H): 12.53 \text{ (1H, s, NH indole), 9.11 \text{ (1H, s, NH), 7.54-6.70 \text{ (4H, m, ArH), 5.35 \text{ (2H, m, CH=CH), 4.18 \text{ (1H, m, CH}_2\text{OH), 2.74 \text{ (2H, t, J = 7.57 Hz, \text{CH}_2\text{CO), 2.23 \text{ (4H, m, CH}_2\text{CH=CHCH}_2, 1.70 \text{ (1H, m, CH}_2\text{OH), 1.56 \text{ (2H, m, CH}_2\text{CH}_2\text{CO), 1.28 \text{ (18H, br.s, (CH}_2\text{)}_{9} \text{ chain), 0.73 \text{ (3H, dist.t, CH}_3).}}\]

[104]
Hydrazono-isatin and N-methyl isatin derivatives

\[ ^{13}C\text{NMR (CDCl}_3, \delta \text{):} \ 175.0, 172.6, 171.0, 142.3, 142.1, 133.9, 130.7, 130.0, 126.2, 124.9, 69.9, 39.5, 36.4, 33.3, 33.0, 32.4, 31.3, 30.8, 29.6, 29.4, 29.1, 28.9, 28.7, 28.3, 28.2, 14.0. \]

\[ \text{MS (ESI): } m/z = 464.319 \text{ found } [M+Na]^+, \text{ calculated } [M+Na]^+ = 464.527. \]

**3-[(9'R, 12'Z)-9'-Hydroxyoctadec-12'-enoyl hydrazono] isatin, (148)**

Yellow solid, yield: 78%; m.p.: 88-89°C

\[ \text{IR (KBr, cm}^{-1}) : 3422 \text{ (O-H stretching), } 3340 \text{ (hetero N-H stretching), } 3100 \text{ (amide N-H stretching), } 2921 \text{ (C-H stretching), } 1685, 1660 \text{ (C=O stretching), } 1580 \text{ (C=N stretching).} \]

\[ ^{1}H\text{NMR (CDCl}_3, \delta \text{H):} \ 12.00 \text{ (1H, s, NH indole), } 9.51 \text{ (1H, s, NH), } 7.22-6.45 \text{ (4H, m, ArH), } 5.32 \text{ (2H, m, CII=CH)}, 4.20 \text{ (1H, m, CHOII)}, 2.71 \text{ (2H, t, } J = 7.45 \text{ Hz, CH}_2\text{CO)}, 2.10 \text{ (4H, m, } CH_2\text{CH}=\text{CHCH}_2\text{)}, 1.75 \text{ (1H, m, CHOII)}, 1.45 \text{ (2H, m, } CH_2\text{CH}_2\text{CO)}, 1.27 \text{ (18H, br.s, (CH}_2\text{)}_9\text{chaine}), 0.77 \text{ (3H, dist.t, } CH_3). \]

\[ ^{13}C\text{NMR (CDCl}_3, \delta \text{C):} \ 176.2, 173.8, 172.7, 143.3, 141.3, 134.2, 132.5, 131.8, 127.9, 70.1, 39.3, 35.9, 33.9, 33.6, 33.2, 32.7, 31.6, 31.6, 29.9, 29.7, 29.4, 29.0, 28.8, 28.6, 28.4, 14.2. \]

\[ \text{MS (ESI): } m/z = 464.408 \text{ found } [M+Na]^+, \text{ calculated } [M+Na]^+ = 464.527. \]

**3-(Hexadeconoyl hydrazono)-N-methyl isatin, (149)**

Orange powder; yield: 80%; m.p.: 89-91°C

\[ \text{IR (KBr, cm}^{-1}) : 3071 \text{ (N-H stretching), } 2921 \text{ (C-H stretching), } 1682, 1651 \text{ (C=O stretching), } 1575 \text{ (C=N stretching).} \]

\[ ^{1}H\text{NMR (CDCl}_3, \delta \text{H):} \ 9.40 \text{ (1H, s, NH), } 7.58-6.85 \text{ (4H, m, ArH)}, 3.28 \text{ (3H, s, } CH_3), 2.80 \text{ (2H, t, } J = 7.52 \text{ Hz, } CH_2\text{CO)}, 1.72 \text{ (2H, m, } CH_2\text{CH}_2\text{CO)}, 1.30 \text{ (24H, br.s, (CH}_2\text{)}_2\text{chaine), 0.87 (3H, dist.t, } CH_3). \]
**Hydrazono-isatin and N-methyl isatin derivatives**

\[ ^{13}C \text{NMR (CDCl}_3, \delta_{C}) : 176.5, 171.0, 161.4, 160.1, 131.2, 130.9, 123.5, 123.1, 121.8, 36.0, 33.4, 31.8, 31.7, 29.5, 29.3, 29.0, 28.9, 25.6, 25.3, 24.4, 23.6, 23.2, 22.8, 22.4, 14.0. \]

**MS (ESI):** m/z = 436.390 found [M+Na]+, calculated [M+Na]⁺ = 436.517.

3-(Undec-10'-enoyl hydrazono)-N-methyl isatin, (150)

Orange powder; yield: 75%; m.p.: 85-86°C

**IR (KBr, cm⁻¹):** 3047 (N-H stretching), 2923 (C-H stretching), 1698, 1660 (C=O stretching), 1598 (C=N stretching).

\[ ^1H \text{NMR (CDCl}_3, \delta_{H}) : 9.42 (1H, s, NH), 7.57-6.87 (4H, m, ArH), 5.80 (1H, tdd, J_H-H_2 = 6.4 Hz, J_H-H_2 = 10.3 Hz, J_H-N = 17.1 Hz, CH_2=CH), 4.99 (1H, dd, J_H-H_2 = 10.3 Hz, J_H-N = 1.3 Hz, H_2C=CH), 3.23 (3H, s, CH_3), 2.82 (2H, t, J = 7.60 Hz, CH_2CO), 2.02 (2H, m, CH_2=CH-CH_2), 1.74 (2H, m, CH_2CH_2CO), 1.32 (10H, br.s, (CH_2)_10 chain). \]

\[ ^{13}C \text{NMR (CDCl}_3, \delta_{C}) : 176.5, 170.1, 161.5, 160.4, 136.3, 132.2, 131.4, 130.1, 123.7, 123.6, 121.5, 35.7, 33.6, 31.0, 29.6, 29.4, 25.9, 24.4, 22.9, 22.6. \]


3-[(9'E)-Octadec-9'-enoyl hydrazono]-N-methyl isatin, (151)

Orange powder; yield: 80%; m.p.: 83-85°C

**IR (KBr, cm⁻¹):** 3089 (N-H stretching), 2924 (C-H stretching), 1668, 1646 (C=O stretching), 1598 (C=N stretching).

\[ ^1H \text{NMR (CDCl}_3, \delta_{H}) : 9.96 (1H, s, NH), 7.50-6.92 (4H, m, ArH), 5.40 (2H, m, CH=CH), 2.78 (2H, t, J = 7.56 Hz, CH_2CO), 2.00 (4H, m, CH2CH=CHCH_2), 1.65 (2H, m, CH_2CH_2CO), 1.28 (20H, br.s, (CH_2)_10 chain), 0.88 (3H, dist.t, CH_3). \]
Hydrazone-isatin and N-methyl isatin derivatives

\[^{13}\text{C NMR (CDCl}_3, \delta)\]: 177.0, 172.4, 160.8, 159.4, 134.8, 133.6, 133.4, 128.9, 128.5, 123.8, 123.4, 35.0, 32.8, 32.4, 32.0, 30.9, 30.4, 30.2, 29.9, 29.8, 29.4, 28.7, 28.0, 25.4, 22.3, 22.0, 13.9.

\(\text{MS (ESI)}: \text{m/z} = 462.230 \text{ found } [M+Na]^{+}, \text{ calculated } [M+Na]^{+} = 462.551.\)

3-[(9'Z, 12'R)-12'-Hydroxyoctadec-9'-enoyl hydrazone]-N-methyl isatin, (152)

Orange sticky oil; yield: 70%

\(\text{IR (KBr, cm}^{-1}\): 3405 (O-H stretching), 3094 (N-H stretching), 2926 (C-H stretching), 1684, 1662 (C=O stretching), 1599 (C=N stretching).

\(^1\text{H NMR (CDCl}_3, \delta): 9.45 (1\text{H, s, NH}), 7.60-6.84 (4\text{H, m, ArH}), 5.41 (2\text{H, m, CH=C=CH}), 4.09 (1\text{H, m, CHO}), 3.23 (3\text{H, s, CH}_3) 2.81 (2\text{H, t, } J = 7.57 \text{ Hz, CH}_2\text{CO}), 2.17 (4\text{H, m, CH}_2\text{CH=CHCH}_2), 1.74 (1\text{H, m, CHOH}), 1.62 (2\text{H, m, CH}_2\text{CH}_2\text{CO}), 1.28 (18\text{H, br.s, (CH}_2)_9 \text{ chain}), 0.89 (3\text{H, dist.t, CH}_3).

\[^{13}\text{C NMR (CDCl}_3, \delta)\]: 176.4, 173.9, 161.5, 160.8, 143.3, 134.0, 133.1, 132.5, 131.4, 125.3, 123.6, 71.9, 37.4, 36.8, 33.9, 31.8, 31.6, 31.4, 30.6, 30.0, 29.9, 29.8, 29.7, 29.1, 28.4, 27.3, 14.0.

\(\text{MS (ESI)}: \text{m/z} = 478.419 \text{ found } [M+Na]^{+}, \text{ calculated } [M+Na]^+ = 478.550.\)

3-[(9'R, 12'Z)-9'-Hydroxyoctadec-12'-enoyl hydrazone]-N-methyl isatin, (153)

Orange sticky oil; yield: 65%

\(\text{IR (KBr, cm}^{-1}\): 3395 (O-H stretching), 3090 (N-H stretching), 2923 (C-H stretching), 1679, 1658 (C=O stretching), 1593 (C=N stretching).

\(^1\text{H NMR (CDCl}_3, \delta): 9.30 (1\text{H, s, NH}), 7.49-6.69 (4\text{H, m, ArH}), 5.40 (2\text{H, m, CH=C=CH}), 4.28 (1\text{H, m, CHO}), 3.15 (3\text{H, s, CH}_3) 2.75 (2\text{H, t, } J = 7.53 \text{ Hz, CH}_2\text{CO}), 2.20 (4\text{H, m, CH}_2\text{CH=CHCH}_2), 1.78 (1\text{H, m, CHOH}), 1.68 (2\text{H, m, CH}_2\text{CH}_2\text{CO}), 1.30 (18\text{H, br.s, (CH}_2)_9 \text{ chain}), 0.76 (3\text{H, dist.t, CH}_3).\)
13C NMR (CDCl3, δ): 175.8, 173.6, 162.5, 161.0, 142.4, 133.2, 133.1, 132.8, 131.3, 124.3, 123.8, 71.6, 36.1, 36.0, 33.7, 31.8, 31.5, 31.2, 30.5, 30.1, 29.9, 29.6, 29.4, 29.1, 28.3, 27.5, 14.1.


4.4.2. Biology

4.4.2.1. Chemicals used in studies

MTT was purchased from Affymetrix (USB), PI was purchased from Molecular Probes (Eugene, OR, USA), anti-rabbit PARP-1/2 (Santa Cruz Biotechnology, CA, USA), monoclonal anti-mouse alpha tubulin (Sigma, St. Louis, MO, USA).

4.4.2.2. Human cancer cell lines and culture

Hep G2, U2OS, HeLa and MCF 7 cells were obtained from American Type Culture Collection (Manassas, VA). They were cultured in RPMI-1640 or DMEM medium along with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Cells were maintained in humidified incubator at 37°C in 5% CO₂-95% air.

4.4.2.3. MTT assay

The cells were incubated with tested compounds at different concentrations for 72 hours at 37°C in a 96 well plate in triplicates. After incubation, MTT dye (5 mg/mL in PBS) was added to each well and kept it for 2 hours at 37°C until blue coloured product developed. Then extraction buffer (20% SDS in 50% dimethylformamide, DMF) was added and incubates it for overnight. The absorbance was measured at 570 nm by multiscanner autoreader (Coda, Bio Rad). The absorption values were expressed as the cell viability (%), according to the control group as 100%. The
concentration required for 50% inhibition of cell viability (IC50) was calculated using the software "Prism 3.0".

4.4.2.4. Flow Cytometry (FACS)

0.5 X 10^6 Hela cells were treated with different concentrations of the test compounds for 24 hours. After an incubation period, cells were harvested, washed with phosphate buffer saline (PBS), and stained with PI (2 µg/mL in PBS) for 2 minutes at room temperature. Under these conditions, live cells exclude dye, and only nonviable cells are stained. Live versus dead cells were analyzed by flow cytometry (BD FACS Aria III).

4.4.2.5. PI Nuclear Staining

For PI staining, cells were fixed in 80% methanol for 6 hours, washed with PBS and then stained with PI (25 µg/mL in PBS). Fluorescence microscopy was done to check nuclear integrity.

4.4.2.6. Western Blotting Protocol

1 X 10^6 Hela cells were cultured on six 60 mm culture dishes (corning) and then was treated with increasing concentration of compounds (148 and 153) (10 and 20 µg/mL) and whole cell extract was prepared by the following method. Briefly, cells were suspended in lysis buffer (1M HEPES, 1M Nacl, 10% NP-40, 0.5M EDTA) PMSF (1 mM), DTT (10 µg/mL), aprotinin (5 µg/mL) and leupeptin (5 µg/mL). The suspension was vortexed five times in interval of 10 minutes and was centrifuged at 14,000 rpm for 10 minutes and supernatant was collected in a chilled fresh microfuge tubes. The 50 µg of protein from each cell extracts were resolved in a denaturing 12% SDS-PAGE gel and after completion of the run, the gel was over laid on a nitrocellulose paper cut to the size of gel and kept in the blotting cassette in the presence of blotting buffer. Finally, the cassette was put in the mini transblot...
apparatus (Bio Rad) and blotting was done for 3 hours at a constant voltage of 80 V. After that, the membrane was taken out and rinsed in Phosphate Buffer Saline-Tween, (PBS-T) for 5 minutes by gentle shaking. Later, the membrane was immersed in 5% non-fat milk solution in PBS-T with gentle shaking for 1 hour at 37°C. The membrane was washed off from the traces of the fat free milk with PBS-T and the membrane was overlaid with primary antibody (anti-PARP 1/2) diluted in PBS-T (1:5000) for 3 hours at shaking. After incubation the membrane was washed with PBS-T thrice for 5 minutes each and layered with anti-rabbit secondary antibody (conjugated with horse-radish peroxidase) diluted in 5% fat free milk solution in PBS-T (1:5000) and incubated for 45 minutes at room temperature. The membrane was washed thrice after incubation and processed for the protein bands of interest using ECL-plus detection reagent followed by development of the bands using X-ray film (Hyperfilm-ECL, Amersham Biosciences). To ensure equal loading of extracted protein, the blot was reprobed with antitubulin antibody.
4.5. References

Hydrazono-isatin and N-methyl isatin derivatives


