Synthesis and cytotoxicity evaluation of 2-Phenyl-1H-benzo[de]isoquinoline-1,3(2H)-dione derivatives as apoptosis inducers with probable anticancer effects

Alireza Aliabadi¹,², Jino Yousefbeigi²,³, Ahmad Mohammadi-Farani¹,⁴, Sahar Jamshidy Navid⁴

¹Pharmaceutical Sciences Research Center, School of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran
²Department of Medicinal Chemistry, Faculty of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran
³Students Research Committee, Kermanshah University of Medical Sciences, Kermanshah, Iran
⁴Department of Pharmacology, Toxicology and Medical Services, Faculty of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran

Abstract
Due to the resistance and exertion of severe side effects of current anticancer drugs, development of new anticancer agents is one of the main goals in medicinal chemistry. Apoptosis process is occurred in all organisms to control their cell numbers and to omit extra or damaged cells. Caspases are an enzyme family that precedes apoptosis. Activation of caspases results in irreversible apoptotic cell death. Naphthalimide derivatives as DNA intercalators have exhibited high anticancer activities against various cell lines. Some naphthalimides such as amonafide have demonstrated remarkable potency in clinical trials. In the current work, a new series of naphthalimide-based anticancer agents were synthesized. Then, the cytotoxic was evaluated by MTT assay in vitro. Three cancerous cell lines were utilized namely AGS (human gastric carcinoma), HT29 (colorectal cancer), PC3 (prostate cancer) and the obtained results were compared to the doxorubicin as reference drug. In order to study the structure activity relationships of the target compounds, various substituents such as Cl, F, NO₂ and -OCH₃ were introduced on the phenyl ring. Generally, electron withdrawing substituents caused better anticancer activity compared to doxorubicin. Some selected derivatives activated the caspase 3 more than control drug and also influenced on mitochondrial membrane potential (MMP) and reduced it. It means that these compounds may induce the apoptosis via the intrinsic pathway. Production of intracellular reactive oxygen species (ROS) was also investigated and the obtained results showed that the most of the tested compounds induced the production of free radicals less than control drug.

Keywords: Synthesis, Naphthalimde, Anticancer, Caspase.

1. Introduction

Unfortunately, the statistics have showed a significant increase in cancer incidence. Besides, the current anticancer therapeutics could cause severe adverse reactions such as gastrointestinal side effects as well as bone marrow toxicity. All the mentioned reasons concomitantly with tumor resistance to the conventional anticancer drugs are motivating factors in medicinal chemistry for investigation towards the discovery of new anticancer drugs [1-4]. Apoptosis or programmed cell death is occurred in all organisms to control their cell numbers and to omit extra or damaged cells. The term ‘apoptosis’ was originally coined by Kerr et al. (1972) for explanation of a distinct morphological alterations in cell related to normal programmed cell death and certain pathological phenomenon in vivo. Cell shrinkage, loss of contact with neighboring cells, formation of cytoplasmic vacuoles, plasma and nuclear membrane blebbing and chromatin condensation are the main changes in an apoptosis process. The mechanism of apoptosis involves a cascade of initiator and effector caspases that are activated sequentially. Caspases as cysteine
proteolytic enzymes known are an enzyme family that precedes apoptosis. Activation of these protease enzymes which are normally present inside the cells in the form of inactive zymogens, lead to the cleavage of multiple protein substrates inside cells and result in irreversible apoptotic cell death. Within the caspase family, caspase 3 has been identified as one of the key effector caspases that cleave multiple protein substrates in cells, and leading to irreversible cell death. Caspases 8 and 9 also have pivotal role in extrinsic and intrinsic pathway of apoptosis respectively. In addition, several clinically used cytotoxic agents, including paclitaxel, docetaxel and vinca alkaloids, are known to primarily act by inducing apoptosis in cancer cells [5-11].

Naphthalimide derivatives, first discovered by Brana and co-workers, have been known as DNA intercalators and exhibited high anticancer activities against various cell lines. Some naphthalimide-based compounds, such as amonafide, mitinafide, elinafide and bisnafide (Figure 1) have demonstrated remarkable potency in clinical trials. However, the obtained results in trials were associated with severe side effects. The study of structure activity relationship revealed that naphthalimide core should be intact while adding other functional groups may decrease the systemic toxicity. Fusion of some aromatic rings like benzene, imidazole, pyrazine, furan and thiophene was carried out to naphthalene nucleus that led to the significant improvement in cellular cytotoxic activity compared to amonafide. Besides, other series of naphthalimides with unfused benzene or furan ring was also investigated that displayed favourable cytotoxicity in cancerous cell lines. Amonafide as naphthalimide-based anticancer agents acts also as topo II poison and is in phase III clinical trials for the treatment of acute myeloid leukemia [12-20].

Based on the new reports about the naphthalimide derivatives as potential anticancer agents, we decided to synthesize a new series of this family towards the development of new lead compounds.

2. Results and Discussion
2.1. Cytotoxicity
A new series of naphthalimide-based anticancer agents were synthesized. The cytotoxic potentiality of these compounds was evaluated by MTT assay in vitro. Three cancerous cell lines were utilized to explore the cytotoxicity effects namely AGS (human gastric carcinoma), HT29 (colorectal cancer), PC3 (prostate cancer) and the obtained results were compared to the doxorubicin as reference drug. In order to study the structure activity relationships of the target compounds, various substituents such as Cl, F, NO₂ and -OCH₃ were introduced on the phenyl ring. According to Table 1, chlorinated derivatives (3a, 3b, 3c) did not show any acceptable potency against
AGS cell line, whereas a significant cytotoxicity was observed while tested on HT29 and PC3 cells. Chlorine moiety rendered a higher potency toward AGS cell line (IC\textsubscript{50} = 15.00 µM) when substituted at position meta compared to other positions of the phenyl ring. Meta positioning of the chlorine moiety was also favorable for cytotoxicity against PC3 cell line. HT29 and PC3 cells demonstrated an outstanding susceptibility to compound 3a with IC\textsubscript{50} = 3.87 and 6.43 µM respectively, while ortho positioning of the chlorine substituent was made. It is notable that compound 3a exhibited superior cytotoxicity than doxorubicin (IC\textsubscript{50} = 7 µM) against HT29 cells. Substitution of the fluorine atom on the phenyl ring caused a remarkable enhancement in activity. All three possible positions of the phenyl ring were suitable for fluorine moiety to increase the potency (ortho > para > meta). It means that increasing in electron negativity and electron withdrawing property of the corresponding moiety could be a beneficial parameter for cytotoxicity. PC3 and AGS cells were more sensitive to compounds 3d, 3e and 3f in comparison with HT29 cells. A contrasting effect was observed for HT29 cell line. In other word, meta positioning of the fluorine atom was more effective than other positions. It means that electron withdrawing property of the moiety on the phenyl residue is a detrimental activity. Nitratated derivatives (3g, 3h, 3i) were so cytotoxic against HT29 as well as PC3 cell lines. All positions of the phenyl ring were beneficial for exertion of the cytotoxicity of the nitro moiety against HT29 cells especially meta position (IC\textsubscript{50} = 1.93 µM). About the PC3 cells, only positions meta and para were suitable for nitro substitution and ortho positioning of this moiety did not cause a remarkable potency. Methoxylated derivatives (3j, 3k, 3l) were also prepared to investigate the role of electron donating property on the phenyl residue. HT29 cell line was more sensitive to the methoxylated compounds in comparison with other cell lines. Electron releasing feature of the methoxy substituent was an enhancing effect of the cytotoxicity in PC3 cells (para > meta > ortho). HT29 and AGS cell lines were also sensitive to the compound 3l with para methoxy group. It is interesting that compound 3l with para methoxy group was the most active derivatives against AGS cell line in this series (IC\textsubscript{50} = 2.12 µM). Utilization of the phenyl residue free of substituent led to an outstanding decline in activity. It seems that presence of a moieties on the phenyl ring especially with electron withdrawing property is necessary for cytotoxic activity.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R</th>
<th>AGS</th>
<th>HT29</th>
<th>PC3</th>
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<tbody>
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<td>36.69</td>
<td>3.87</td>
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<tr>
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<td>3-Cl</td>
<td>15.00</td>
<td>46.73</td>
<td>5.47</td>
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<tr>
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<td>4-Cl</td>
<td>&gt;100</td>
<td>11.02</td>
<td>29.87</td>
</tr>
<tr>
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<td>73.96</td>
<td>8.37</td>
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<tr>
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<td>3-F</td>
<td>6.91</td>
<td>8.24</td>
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<td>4-F</td>
<td>5.25</td>
<td>115.3</td>
<td>4.92</td>
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<td>3g</td>
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<td>4.73</td>
<td>59.97</td>
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<td>3-NO\textsubscript{2}</td>
<td>15.31</td>
<td>1.93</td>
<td>4.87</td>
</tr>
<tr>
<td>3i</td>
<td>4-NO\textsubscript{2}</td>
<td>&gt;100</td>
<td>4.23</td>
<td>5.47</td>
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<td>2-OCH\textsubscript{3}</td>
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<td>15.51</td>
<td>10.30</td>
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<td>9.57</td>
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<tr>
<td>3m</td>
<td>H</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

| Doxorubicin | - | 0.9 | 7 | 4.6 |

2.2. Caspase 3 activity

The effect of some selected compounds was evaluated on activity of caspase 3. According to the obtained cytotoxicity results from the MTT assay, compounds 3a, 3b, 3d, 3e, 3f, 3h and 3l were chosen and their impact on the caspase 3 activation were investigated. The obtained data were compared to doxorubicin as control drug (Figure 2). The tested derivatives enhanced the caspase 3 activity more than doxorubicin except for compounds 3e and 3h. Compounds 3e and 3h caused an increase in caspase 3 activity equal to doxorubicin. It could be bear in mind that cytotoxicity of the tested compounds maybe made via activation of the caspase 3 pathway.
2.3. Mitochondrial Membrane Potential

Some selected compounds were investigated for changes in mitochondrial membrane potential. According to Figure 3, compounds 3b, 3d and 3e decreased the membrane potential of mitochondria compared to doxorubicin as control drug. It seems that these compounds induce the apoptosis process via the intrinsic pathway, whereas the other examined derivatives namely 3a, 3f, 3h and 3l did not cause any significant reduction in membrane potential of mitochondria. In other word, the later derivatives were not capable to induce the intrinsic pathway of apoptosis. But according to Figure 2, these compounds increased the activity of caspase 3. It could be suggested that these compounds induce the apoptosis through intrinsic pathway.

2.4. Intracellular Reactive Oxygen Species (ROS) Measurement

The production of intracellular reactive oxygen species was measured to investigate the role of free radicals in the induction of apoptosis. Because free radical generation would led to the DNA damage and eventually end to the apoptosis induction. According to the Figure 4, none of the tested compounds caused effective generation of reactive oxygen species more than control drug except for compound 3l. Only compound 3l produced ROS more than the control.
3. Experimental

3.1. Chemistry

The corresponding chemical reagents and starting materials were purchased from the commercial companies such as Merck and Sigma-Aldrich. The purification of the prepared compounds was carried out by column chromatography using ethyl acetate/petroleum ether. Spectroscopic methods were applied for characterization of the synthesized compounds. 1H NMR spectra were acquired by Bruker 500 MHz in deuterated dimethylsulfoxide (DMSO-d$_6$) and the obtained data were expressed as δ (ppm) compared to tetramethylsilane (TMS) as internal standard. Infrared (IR) spectra of the prepared compounds were obtained by Shimadzu 470 with preparing potassium bromide (KBr) disk. The mass spectra were run on a Finigan TSQ-70 spectrometer (Finigan, USA) at 70 eV. Melting points were determined using electrothermal 9001 melting point analyzer apparatus and are uncorrected.

3.2. General procedure for synthesis of compounds 3a-3m

In a flat bottom flask, 0.2 g (1 mmole) naphthalene-1,8-dicarboxylic anhydride was refluxed equivalently with appropriate aniline derivatives in 20 ml toluene. Reflux condition was carried out for 24 h and the end point of the reaction was determined by thin layer chromatography. The toluene was evaporated under reduced pressure and water/ethyl acetate (20/20 ml) was added to the residue. Aqueous phase was discarded and organic phase was washed two times by diluted sulfuric acid (2 %) and brine. Ethyl acetate was evaporated and the obtained powder was purified using column chromatography (ethyl acetate/petroleum ether, 60/40) [21-24]. The final target derivatives 3a-3m were prepared with a moderate yields (Table 2).

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Chemical Formula</th>
<th>MW (g/mol)</th>
<th>mp (°C)</th>
<th>Yield (%)</th>
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<td>3a</td>
<td>2-Cl</td>
<td>C$<em>{18}$H$</em>{10}$ClNO$_2$</td>
<td>307</td>
<td>263</td>
<td>49</td>
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<td>307</td>
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<td>60</td>
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<td>3-F</td>
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<td>4-F</td>
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<td>3m</td>
<td>H</td>
<td>C$<em>{18}$H$</em>{14}$NO$_2$</td>
<td>273</td>
<td>176</td>
<td>60</td>
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</table>

2-(2-Chlorophenyl)-1H-benzo(de)isoquinoline-1,3(2H)-dione (3a)

$^1$H NMR (CDCl$_3$, 500 MHz) δ (ppm): 7.34 (t, 1H, J = 10 Hz, H$_2$-2-chlorophenyl), 7.39 (t, 1H, H$_2$-2-chlorophenyl), 7.48 (d, 2H, J = 10 Hz, H$_3$-2-chlorophenyl), 7.67 (d, 2H, J = 10 Hz, H$_3$-2-chlorophenyl), 7.86 (m, 2H, H$_5$-naphthalimide), 8.35 (dd, 2H, H$_6$-naphthalimide), 8.69 (t, 2H, J = 10 Hz, H$_5$-naphthalimide). IR (KBr, cm$^{-1}$) ν: 3444, 1770, 1739, 1577, 1537.

2-(3-Chlorophenyl)-1H-benzo(de)isoquinoline-1,3(2H)-dione (3b)

$^1$H NMR (CDCl$_3$, 500 MHz) δ (ppm): 7.28 (d, 1H, J = 10 Hz, H$_2$-3-chlorophenyl), 7.39 (s, 1H, H$_2$-3-chlorophenyl), 7.52 (m, 2H, J = 10 Hz, H$_5$-3-chlorophenyl), 7.86 (m, 2H, H$_5$-naphthalimide), 8.35 (dd, 2H, H$_6$-naphthalimide), 8.69 (t, 2H, J = 10 Hz, H$_5$-naphthalimide). IR (KBr, cm$^{-1}$) ν: 3444, 1774, 1735, 1670, 1585.

2-(4-Chlorophenyl)-1H-benzo(de)isoquinoline-1,3(2H)-dione (3c)

$^1$H NMR (CDCl$_3$, 500 MHz) δ (ppm): 7.31 (d, 2H, J = 10 Hz, 4-chlorophenyl), 7.56 (d, 2H, J = 10 Hz, 4-chlorophenyl), 7.86 (m, 2H, H$_5$-naphthalimide), 8.33 (d, 1H, H$_6$-naphthalimide), 8.37 (d, 1H, H$_7$-naphthalimide), 8.88 (s, 1H, H$_3$-naphthalimide), 3.70 (s, 3H, OCH$_3$).
8.69 (m, 2H, H₃₂-naphthalimide). IR (KBr, cm⁻¹): 3448, 3066, 1774, 1735, 1658, 1581. MS (m/z, %): 309 (M⁺+2, 30), 307 (M⁺, 90), 262 (10), 228 (15), 198 (40), 180 (60), 154 (75), 126 (100), 99 (15), 74 (30), 63 (30).

2-(2-Fluorophenyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (3d)

¹H NMR (CDCl₃, 500 MHz) δ (ppm): 7.29 (d, 1H, J = 10 Hz, H₄₂-2-fluorophenyl), 7.32 (d, 1H, J = 10 Hz, H₆₂-fluorophenyl), 7.33 (t, 1H, J = 10 Hz, H₅₂-fluorophenyl), 7.38 (t, 1H, J = 10 Hz, H₃₂-2-fluorophenyl), 7.82 (m, 2H, H₈₂-naphthalimide), 8.30 (dd, 2H, H₆,₇-naphthalimide), 8.65 (t, 2H, J = 10 Hz, H₅,₈₉-naphthalimide). IR (KBr, cm⁻¹) ν: 3444, 3070, 1774, 1735, 1670, 1585, 1504, 1375.

2-(3-Fluorophenyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (3e)

¹H NMR (CDCl₃, 500 MHz) δ (ppm): 7.23-7.41 (m, 2H, J = 10 Hz, 2-fluorophenyl), 7.51-7.65 (m, 1H, J = 10 Hz, H₆₂-2-fluorophenyl), 7.82 (m, 2H, H₈₂-naphthalimide), 8.30 (dd, 2H, H₆,₇-naphthalimide), 8.65 (t, 2H, J = 10 Hz, H₅,₈₉-naphthalimide). IR (KBr, cm⁻¹) ν: 3448, 3070, 1774, 1735, 1674, 1581, 1307.

2-(4-Fluorophenyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (3f)

¹H NMR (CDCl₃, 500 MHz) δ (ppm): 7.28 (t, 2H, J = 10 Hz, H₃₂-4-fluorophenyl), 7.35 (t, 2H, J = 10 Hz, H₃₂-4-fluorophenyl), 7.87 (m, 2H, H₈₂-naphthalimide), 8.35 (dd, 1H, H₆,₇-naphthalimide), 8.69 (dd, 1H, H₆,₇-naphthalimide). IR (KBr, cm⁻¹) ν: 3421, 3074, 1774, 1735, 1662, 1585, 1508, 1354, 1307, 1234. MS (m/z, %): 291 (M⁺, 100), 246 (60), 180 (90), 152 (25), 126 (60).

2-(2-Nitrophenyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (3g)

¹H NMR (CDCl₃, 500 MHz) δ (ppm): 6.75 (t, 1H, J = 10 Hz, H₄₂-2-nitrophenyl), 6.85 (d, 1H, J = 10 Hz, H₆₂-nitrophenyl), 7.40 (t, 1H, J = 10 Hz, H₅₂-nitrophenyl), 7.87 (d, 2H, H₈₂-naphthalimide), 8.16 (d, 1H, J = 10 Hz, H₆₂-nitrophenyl), 8.37 (t, 2H, H₆,₇-naphthalimide), 8.69 (d, 2H, H₆,₈₉-naphthalimide). IR (KBr, cm⁻¹) ν: 3475, 3352, 1770, 1739, 1627, 1577, 1508, 1431, 1346.

2-(3-Nitrophenyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (3h)

¹H NMR (CDCl₃, 500 MHz) δ (ppm): 6.95 (d, 1H, J = 10 Hz, H₆₂-2-nitrophenyl), 7.28 (t, 1H, J = 10 Hz, H₅₂-nitrophenyl), 7.49 (s, 1H, H₂-2-nitrophenyl), 7.59 (d, 1H, J = 10 Hz, 3-nitrophenyl), 7.84 (t, 2H, H₈₂-naphthalimide), 8.33 (d, 2H, H₆,₇-naphthalimide), 8.64 (d, 2H, H₆,₈₉-naphthalimide). IR (KBr, cm⁻¹) ν: 3452, 3371, 1770, 1739, 1581, 1523, 1350.

2-(4-Nitrophenyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (3i)

¹H NMR (CDCl₃, 500 MHz) δ (ppm): 6.62 (d, 2H, J = 10 Hz, H₃₂-4-nitrophenyl), 7.84 (t, 2H, H₈₂-naphthalimide), 8.06 (d, 2H, J = 10 Hz, H₃₂-4-nitrophenyl), 8.33 (d, 2H, H₆,₇-naphthalimide), 8.64 (d, 2H, H₆,₈₉-naphthalimide). IR (KBr, cm⁻¹) ν: 3479, 3360, 3066, 1770, 1739, 1581, 1303. MS (m/z, %): 318 (M⁺, 10), 198 (60), 154 (100), 126 (85), 98 (10), 87 (10), 74 (15), 63 (15).

2-(2-Methoxyphenyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (3j)

¹H NMR (CDCl₃, 500 MHz) δ (ppm): 3.82 (s, 3H, -OCH₃), 7.15 (m, 1H, 2-methoxyphenyl), 7.31 (t, 1H, H₅₂-methoxyphenyl), 7.83 (t, 1H, H₆₂-methoxyphenyl), 7.88 (t, 2H, H₈₂-naphthalimide), 8.30 (d, 1H, J = 10 Hz, H₆₂-methoxyphenyl), 8.37 (d, 2H, J = 10 Hz, H₆,₇-naphthalimide), 8.68 (d, 2H, J = 10 Hz, H₆,₈₉-naphthalimide). IR (KBr, cm⁻¹) ν: 3444, 3070, 1774, 1735, 1674, 1585.

2-(3-Methoxyphenyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (3k)

¹H NMR (CDCl₃, 500 MHz) δ (ppm): 3.84 (s, 3H, -OCH₃), 6.86 (s, 1H, H₂-3-methoxyphenyl), 6.91 (d, 1H, J = 10 Hz, H₃₂-methoxyphenyl), 7.03 (dd, 1H, J = 10 Hz, 2.5 Hz, H₄₂-3-methoxyphenyl), 7.46 (t, 1H, J = 10 Hz, H₆₂-methoxyphenyl), 7.81 (m, 2H, H₈₂-naphthalimide), 8.27 (d, 1H, J = 10 Hz, H₆,₈₉-naphthalimide), 8.32 (d, 1H, J = 10 Hz, H₆-naphthalimide), 8.65 (t, 2H, H₆,₈₉-naphthalimide). IR (KBr, cm⁻¹) ν: 3444, 3074, 1774, 1735, 1708, 1662, 1662, 1585.

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2-(4-Methoxyphenyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (3I)

1H NMR (CDCl3, 500 MHz) δ (ppm): 7.06 (d, 2H, J = 10 Hz, H_{2,6}-4-methoxyphenyl), 7.23 (d, 2H, J = 10 Hz, H_{2,6}-4-methoxyphenyl), 7.79 (t, 1H, J = 5 Hz, H_{3}-naphthalimide), 7.83 (t, 1H, J = 10 Hz, H_{8}-naphthalimide), 8.26 (d, 1H, J = 10 Hz, H_{6}-naphthalimide), 8.32 (d, 1H, J = 10 Hz, H_{2}-naphthalimide), 8.64 (d, 1H, J = 10 Hz, H_{4,9}-naphthalimide). IR (KBr, cm⁻¹) ν: 3448, 3070, 2927, 1770, 1739, 1670, 1581, 1303. MS (m/z, %): 303 (M⁺, 55), 198 (70), 180 (35), 154 (100), 126 (90).

2-Phenyl-1H-benzo[de]isoquinoline-1,3(2H)-dione (3m)

1H NMR (CDCl3, 500 MHz) δ (ppm): 7.32 (d, 2H, J = 10 Hz, phenyl), 7.48 (t, 1H, J = 10 Hz, phenyl), 7.65 (t, 2H, J = 10 Hz, phenyl), 7.78 (t, 2H, J = 10 Hz, H_{3}-naphthalimide), 8.27 (d, 2H, J = 10 Hz, H_{2}-naphthalimide), 8.64 (d, 1H, J = 10 Hz, H_{4,9}-naphthalimide). IR (KBr, cm⁻¹) ν: 3437, 3066, 1774, 1735, 1662, 1581, 1354, 1234. MS (m/z, %): 273 (M⁺, 90), 272 (100), 228 (40), 180 (40), 152 (15), 126 (40).

3.3. Cytotoxicity evaluation

The in vitro cytotoxic activity of compounds 3a-3m were assessed against three cancerous cell lines consisting of PC3 (Prostate cancer), HT29 (Colon cancer) and AGS (Human gastric carcinoma) using MTT (3-(4,5-dimethylthiaizol-2-yl)-2,5-diphenyl tetrazolium bromide) assay procedure. Briefly, cells (5x10⁵) were seeded in 96-well plates and incubated for 48 h for cell attachment. The cells were then incubated with various concentrations of synthesized compounds 3a-3m for 24 hrs. Cells were washed with PBS and then incubated with MTT (0.5 mg/ml) for 4 hrs at 37°C. Live cells can reduce MTT to blue formazan crystals which are then dissolved in DMSO for 5 minutes. Intensity of the color is directly proportional to the number of viable cells. The absorbance of each well was read in a EL×808 microplate reader (BioTek,USA) at 550 nm. Two independent experiments in triplicate were performed for determination of sensitivity to each compound, the IC₅₀ (µM) were calculated by linear regression analysis, expressed in Mean. Doxorubicin was used as a standard drug.

3.4. Caspase activation assay

The activity of caspase-3 was determined by the sigma colorimetric caspase-3 kit according to manufacturer’s instrument. This assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA in equal amount of cells protein lysate. Briefly, 1x10⁶ cells were collected and lysed with 50 µL of chilled lysis buffer and incubated on ice for 10 min. Cell lysates were centrifuged at maximum speed for 5 min at 4°C, after which 50 µL of 2x reaction buffer / DTT mix and 5 µL of 1 mM caspase-3 substrate (DEVD-pNA) were added to each reaction and incubated at 37°C for 1 h. The pNA light emission was quantified using a microplate reader at 400 or 405-nm. Comparison of the absorbance of pNA from an apoptotic sample with an un-induced control allowed determination of the fold increase in caspase-3 activity. The protein content was determined by the Bio-Rad protein assay kit using the bovine serum albumin as a standard. The above stages were also performed for determining of the activation of caspases 8 and 9 [25].

3.5. Measurement of Mitochondrial Membrane Potential

The cells were cultured in the above mentioned conditions and then rhodamine 123 was added to the media at a final concentration of 25 nM. After 30 min of incubation, the cells were harvested and washed with phosphate-buffered saline (PBS). Cells were lysed by Triton-X 100 and the fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 520 nm using a micro-plate reader (BioTek, H1M, USA). During apoptosis mitochondrial cell membrane is depolarized and rhodamine is lost from the membrane hence intracellular fluorescence intensity is decreased [26].

Intracellular reactive oxygen species (ROS) measurement

Intracellular oxidative stress was assessed by measuring ROS generation using the fluorescent dye DCF-DA [27]. Cells (3x10⁵) were cultured and pretreated with the IC₅₀ concentrations of each compound for 24 hrs. After incubation with 10 µM DCF-DA for 45 min, cells were washed with PBS, and the intensity of the fluorescence was monitored using the excitation and emission wavelengths of 485 nm and 530 nm, respectively.

4. Conclusion

A new series of naphthalimide-based anticancer agents were synthesized and their cytotoxicity were assessed in vitro against AGS (human gastric carcinoma), HT29 (colorectal cancer), and PC3 (prostate cancer) cell
lines. Some of the selected derivatives demonstrated good activation of caspase 3 as well as reduction in mitochondrial membrane potential. Some active compounds such as 3a, 3b, 3e, 3f, 3h and 3l in this project could be proposed as novel anticancer lead compounds. These compounds possessed cytotoxicity potency less than 10 µM and also showed an outstanding property for inducing apoptosis.

Acknowledgement
Authors appreciate from the research council of Kermanshah University of Medical Sciences for financial supports. This work was performed in partial fulfillment of the requirement for PharmD of Ms Jino Yousefbeigi.

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