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In Vitro Molecular Biology Studies of Spirooxindole Heterocyclic Hybrids

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Abstract: In the present report, we provide the results of the molecular biology studies of spiroheterocyclic hybrids, where the derivatives are found to possess potential anticancer activity towards cancer cells. A series of spirooxindole–pyrrolidine heterocyclic hybrids were evaluated for cell viability and proliferation against HepG2 cancer cells at concentrations in the range of 12.5–200 µg/mL over two different time periods of 24 and 48 h. In addition, the highly active compounds were also verified for their behavior towards noncancer cells (L929 cells), and it was found that the tested derivatives were not aggressive due to the observation of only limited cell loss, as compared to the cancer cells. Further analysis of the observed toxicity mechanism showed the apoptotic pathway was mediated by oxidative stress, with the involvement of caspases.

Keywords: functionalized spiroheterocycles; anticancer activity; controlled cell death; apoptosis; ROS generation; caspase-3 activity

1. Introduction

Cancer, an uncontrolled growth of abnormal cells, is the second foremost origin of death worldwide after heart disease [1–3]. Cancer is the cause of nearly 14 million demises every year and this number is predicted to double by the year 2030. Cancerous diseases are mostly complicated in humans and can originate in various organs like breast, colon, lung, stomach, and liver, to mention a few [4]. In order to treat such cancerous diseases, various treatment modalities are available, but each method has its own limitations. Among many different treatment modalities, cancer chemotherapy has proven to be easy and proficient for the eradication of aggressive cancer cells without causing trouble to normal cells at a higher level [5]. For the successful implementation of this chemotherapeutic treatment, the selection and recognition of highly active chemical moieties are of the highest significance because of the determined ability of the tumor cell's immune response towards traditional chemotherapeutic agents. Since multidrug resistance development is one of the major challenges associated with the current chemotherapeutics, continuous research is highly demanded in the oncology sector for the identification and testing of novel chemo-moieties that can offer lesser toxicities and uplifted efficiencies [6,7].

Apoptosis, also called programmed cell death, is a highly preferred mechanism for the control of tumor cells, which generally occurs through oxidative stress and DNA damage when the cells lose their capacity to repair the damage caused by the therapeutic/anticancer drugs. In addition, the repair mechanism associated with the apoptosis process helps to control the aggressive growth and proliferation of unwanted cells [8]. Hence, any defects involved in the apoptotic signaling process may lead to the propagation of various diseases in humans, which mainly includes cancer. The cancer cells that develop in such a way contribute to the growth of unwanted masses, called “tumors”,

by surviving over their intended life spans, subverting them from the need for exogenous survival factors and protecting them from oxidative stress and hypoxia. Thus, the developed tumors maintain the genetically altered cells, which promote angiogenesis, deregulate the normal cell proliferation process, interfere with cell differentiation, and increase the possibilities of invasiveness during the progression stage. Therefore, restoring the natural capacity of diseased cells to easily participate in the normal apoptotic process can be a reliable strategy in potential cancer treatments [9].

Spiropyrrolidine-oxindole molecular scaffolds hold interesting structural features and robust bioactivity profiles, including antibacterial [10], antiviral [11], anticancer [12–14], and local anesthetic activities [15], and have ensued as promising synthetic targets. The activity of such central ring systems for the exploration and exploitation of pharmacophore space via diversity-oriented synthesis (DOS) has led to the discovery of new drug candidates [16,17]. For instance, natural spirooxindoles viz. horsfiline [18], and elacomine [19] display important biological activities [20,21]. Notably, even non-natural spirooxindoles, namely, MI-773 and MI-888 (Figure 1), maintain the capacity to control the cell-cycle and protein–protein interactions associated with the nonpeptidic inhibitors of p53–MDM2, which, in general, is considered as dangerous for the p53 protein's activity of tumor-suppression. Further, inhibiting the MDM2–p53 protein interactions can have potential therapeutic implications in cancer treatment [22].

Figure 1. Representative natural and unnatural spirooxindole–pyrrolidine heterocyclic hybrids. (A) The spirooxindole–pyrrolidine heterocyclic hybrids, (B) The N-styryl-substituted spirooxindole–pyrrolidine heterocyclic hybrids, (C) The N-acrolyl derivatives.

In this context, we have been engaged in the exploration of the biological potential of spiropyrrolidine–oxindole heterocyclic hybrids. For instance, the spirooxindole–pyrrolidine heterocyclic hybrids (A) with unsubstituted piperidone moiety have displayed promising anticancer activity in leukemia lymphoblastic (CCRF-CEM), human breast carcinoma (MDA-MB-231), and ovarian carcinoma (SK-OV-3) cells [23]. Additionally, the *N*-styryl-substituted spirooxindole–pyrrolidine heterocyclic hybrids (B) have displayed potent anticancer activities as well [24,25]. Moreover, the *N*-acrylyl derivatives (C) displayed promising cholinesterase inhibitory activities [26]. Furthermore, compounds embedded with *N*-pyridyl structural subunits have been shown to exhibit good-to-excellent anticancer activities against HepG2 cells [27]. It is pertinent to note that the inherent ability of spiroheterocyclic hybrids to project functionality in all three dimensions facilitates easy interactions of ligands with the three-dimensional binding site of spiroheterocyclic hybrids than with planar systems [28,29]. Prompted by this characteristic feature and the abovementioned biological precedents of the spiroheterocyclic hybrids, herein, we present the behavior of spiropyrrolidine–oxindole heterocyclic hybrids derived from *N*-pyridyl-4-piperidone- α,β -unsaturated ketones towards cancer and noncancer cells. We presume the molecular hybridization of spirooxindole, pyrrolidine, piperidone, and *N*-pyridyl units embedded with an α,β -unsaturated ketone moiety into new single hybrid architectures, with preselected activities of the original individual subunits that would enhance the biological profiles of new constructs. In one of our recent studies, we investigated the biological potential of *N*-pyridyl 4-piperidones embedded with an α,β -unsaturated ketone moiety [30]. As a continuation, we present our findings of *N*-pyridyl-substituted spirooxindole–pyrrolidine heterocyclic hybrids constructed through the aforementioned *N*-pyridyl 4-piperidone α,β -unsaturated ketones and their molecular biology activities towards cancer and noncancer cells.

2. Materials and Methods

The selection of cell lines and all the assay protocols for testing the anticancer activity and intracellular mechanisms of spirooxindole–pyrrolidine heterocyclic hybrids were in accordance with our recently published article [30]. The derivatives **1(a–h)** with a range of concentrations (12.5–200 $\mu\text{g/mL}$) were first tested against HepG2 cancer cells over an incubation period of 24–48 h. From those studies, the two most active compounds (**1b** and **1f**) were carried over to test for cell viability and proliferation against the L929 noncancer cell line. Furthermore, the intracellular mechanistic pathways of treatment with highly active compound **1b** were studied against the cancer cell line by means of apoptosis, oxidative stress, and caspase activity assays. For the analysis, melphalan (Mel; 15 $\mu\text{g/mL}$) was used as the positive control, and cells without any treatment were used as the negative control. Additionally, based on statistical analysis, * corresponds to significant values ($p < 0.05$) and ** to highly significant ($p < 0.01$) values against the control measurements. The detailed procedure for the maintenance of cell cultures and assay protocols is provided in the supporting information file.

3. Results and Discussion

The required *N*-pyridyl–spirooxindole–pyrrolidine heterocyclic hybrids **1(a–h)** were synthesized in accordance with our recent study [31]. The molecular structure of these heterocyclic hybrids **1(a–h)** is shown in Scheme 1. Initially, these heterocyclic hybrids **1(a–h)** were tested for their anticancer potential against the HepG2 cancer cell line. The in-vitro cell viability studies, following the incubation of HepG2 cancer cells with that of the test compounds **1(a–h)** at concentrations in the range of 12.5–200 $\mu\text{g/mL}$ over 24 and 48 h, are shown in Figures 2 and 3, respectively.

Scheme 1. Schematic representation of spirooxindole-pyrrolidine heterocyclic hybrids of the present study.

Figure 2. The percentage viability of HepG2 cancer cells when treated with the synthesized derivatives 1(a–h) in the concentration range of 12.5–200 µg/mL and compared with that of positive (melphalan (Mel); 15 µg/mL) and negative controls (no treatment) over a 24 h period. * and ** correspond to significant ($p < 0.05$) and highly significant ($p < 0.01$) values against the untreated control.

Figure 3. The percentage viability of HepG2 cancer cells, followed by the treatment of synthesized derivatives 1(a–h) in the concentration range of 12.5–200 µg/mL and compared against the positive control (Mel; 15 µg/mL) and negative control (no treatment) over a 48 h period. * and ** correspond to significant ($p < 0.05$) and highly significant ($p < 0.01$) values against the untreated control.

From the comparative analysis of the obtained results (Figure 2), all the tested compounds exhibited some level of activity against the cancer cells as we observed a significant number of cell losses starting from 50 µg/mL concentration (48% cell loss) over a 24-h period (as compared against

positive control of Mel and negative control of no treatment). Additionally, with an increase of the incubation period to 48 h (Figure 3), this activity increased (72% cell loss at the same concentration of 50 $\mu\text{g/mL}$) due to increased interaction of the testing compounds with the cells. The IC_{50} values, following the incubation of synthesized compounds **1(a–h)** with that of HepG2 cancer cells for 24 and 48 h, are tabulated in Table 1. From the comparison of IC_{50} values, the least activity was observed for compound **1g** at 24 h and compound **1a** at 48 h incubation periods. However, the highest activity was noted for compound **1b**, followed by compound **1f**, at both the incubation periods of 24 and 48 h. Based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay results, the anticancer activity and the associated IC_{50} values order during the 24 h incubation period is **1b** > **1f** > **1d** > **1h** > **1a** > **1e** > **1c** > **1g**, while for the 48 h period, it is **1b** > **1f** > **1c** > **1g** > **1h** > **1d** > **1e** > **1a**.

Table 1. Comparison of the IC_{50} values of the tested compounds **1(a–h)** against HepG2 cells over 24 and 48 h incubation periods.

Entry	Compound	IC_{50} Values in $\mu\text{g/mL}$ (μM); 24 h		
		HepG2 Cells	HepG2 Cells	L929 Cells
1	1a	237.59 ± 12.5 (385.23 ± 20.27)	126.81 ± 10.4 (205.61 ± 16.86)	-
2	1b	72.24 ± 8.7 (112.03 ± 13.49)	23.81 ± 2.3 (36.92 ± 3.57)	301.72 ± 12.51 (467.93 ± 19.40)
3	1c	265.24 ± 9.4 (391.90 ± 13.89)	29.83 ± 2.1 (44.07 ± 3.10)	-
4	1d	107.74 ± 7.5 (152.44 ± 10.62)	44.1 ± 2.6 (62.40 ± 3.68)	-

Table 1. Cont.

Entry	Compound	IC ₅₀ Values in $\mu\text{g/mL}$ (μM); 24 h		
		HepG2 Cells	HepG2 Cells	L929 Cells
5	1e	237.64 $\pm$ 8.9 (368.55 $\pm$ 13.80)	45.86 $\pm$ 2.8 (71.12 $\pm$ 4.34)	-
6	1f	90.01 $\pm$ 5.6 (131.28 $\pm$ 8.17)	25.01 $\pm$ 3.2 (36.48 $\pm$ 4.67)	257.16 $\pm$ 9.6 (375.06 $\pm$ 14.00)
7	1g	509.28 $\pm$ 14.3 (780.23 $\pm$ 21.91)	40.51 $\pm$ 3.6 (62.06 $\pm$ 5.52)	-
8	1h	157.27 $\pm$ 11.3 (208.43 $\pm$ 14.98)	43.17 $\pm$ 4.5 (57.21 $\pm$ 5.96)	-
9	Mel	-	-	-

The MTT studies (HepG2 cancer cells) for the synthesized derivatives indicated that compounds **1b** and **1f** are highly active at the highest incubation period of 48 h, and hence, we selected these two compounds and assay conditions for the following analysis. Therefore, to test the behavior of highly active compounds **1b** and **1f** on healthy normal cells, the noncancer L929 cells were studied by incubating them at 12.5–200 $\mu\text{g/mL}$ concentration over a 48-h period (Figure 4). From the comparative analysis of the results with that of positive and negative controls, both compounds **1b** and **1f** were not

found to induce significant cell viability losses, as we observed only 18% loss for compound **1b** and 22% loss for compound **1f** at 50 $\mu\text{g/mL}$ concentration. The cells at the highest tested concentration of 200 $\mu\text{g/mL}$ provided approximately 40% loss, indicating that the compounds were behaving in a safe manner towards the noncancer cells while offering anticancer activity against the cancer cells. In addition, the information provided in Table 1 indicates that compound **1b** has the IC_{50} value of 301 $\mu\text{g/mL}$, while compound **1f** has the IC_{50} value of 257 $\mu\text{g/mL}$; these values are quite high compared to the cancer cell IC_{50} values. Thus, taking into consideration such variation in the activity of our synthesized compounds against cancer and noncancer cells, it can be inferred that compounds **1b** and **1f** potentially serve as safe therapeutic agents.

Figure 4. The percentage viability of L929 noncancer cells, followed by the treatment of compounds **1b** and **1f** at concentrations in the range of 12.5–200 $\mu\text{g/mL}$ (48 h period). Positive control of Mel (15 $\mu\text{g/mL}$) and negative control of cells without any treatment were applied. * corresponds to significant ($p < 0.05$) values against the untreated control.

Figure 5 shows the comparison of the flow cytometry of the apoptotic assay results for the highly active compound **1b** at its IC_{50} concentration (24 $\mu\text{g/mL}$; 37 μM) with that of positive control (Mel; 15 $\mu\text{g/mL}$) and negative control (cells without any treatment). From Figure 5, A-1, B-1, and C-1 correspond to the total population of cells used for the fluorescence analysis of the negative control, the positive control (Mel), and compound **1b**, respectively. Similarly, A-2 (untreated control), B-2 (Mel), and C-2 (compound **1b**) correspond to the combined fluorescence of PI and Annexin-V-FITC, while A-3 (untreated control), B-3 (Mel), and C-3 (compound **1b**) correspond to the Annexin-V-FITC fluorescence intensity. From the analysis, it was found that the compound-**1b**-treated cells (Figure 5, C-2) exhibited about 48% live, 6% early-apoptotic, 37% late-apoptotic, and 7% necrotic cells. However, the positive control (Mel)-treated cells (Figure 5, B-2) had 2% early-apoptotic, 46% late-apoptotic, and 34% necrotic cells, while the negative control cells had 97% live cells. The comparison of our compound **1b** result (Figure 5, C-2) with that of positive (Figure 5, B-2) and negative controls (Figure 5, A-2) can primarily indicate the occurrence of a late-apoptotic mechanism. Furthermore, the compound-**1b**-treated cells (Figure 5, C-3) indicated the M1 and M2 values of 37% (normal cells) and 63% (apoptotic cells), similar to the positive control (Figure 5, B-3; 35% normal and 65% apoptotic), and thereby confirm an exclusive apoptotic pathway in the cancer cells.

Figure 5. Comparison of apoptosis assay results for HepG2 cancer cells at 48 h incubation period. From the figure, A, B, and C correspond to the negative control, the positive control (Mel; 15 µg/mL), and compound **1b**, respectively, while 1, 2, and 3 stand for the population of cells selected for the analysis (FSC-H: forward scatter histogram; SSC-H: side scatter histogram), combined fluorescence from PI and Annexin-V-FITC, and pure Annexin-V-FITC fluorescence, respectively.

Figure 6 shows the comparison of ROS assay results provided by flow cytometry for the highly active compound **1b** at its IC₅₀ concentration (24 µg/mL) with that of positive control (Mel; 15 µg/mL) and negative control (cells without any treatment). From the figure, the population of cells tested for the flow cytometric analysis of the ROS assay is represented in Figure 6 of A-1 (negative control), B-1 (positive control), and C-1 (compound **1b**), while the corresponding sample's H₂DCFDA fluorescence population are in A-2, B-2, and C-2, respectively. The H₂DCFDA fluorescence intensities from the negative control, the positive control, and compound **1b** are compared in Figure 6 (A-3, B-3, and C-3, respectively). From the comparison of fluorescence intensities for compound-**1b**-treated cells (Figure 6, C-3), about 10% of cells (M1) were normal and 89% (M2) were experiencing ROS-mediated oxidative stress. This is quite high when compared to the M1 and M2 values indicated by positive-control-treated cells (Figure 6, B-3), i.e., 86% normal and 13% ROS-producing cells (respectively), and, thereby, confirms the oxidative ability of our synthesized compound **1b**.

Figure 7 shows the comparison of the caspase-3 activity of compound **1b** (24 µg/mL concentration) with that of positive control (Mel; 15 µg/mL) and negative control (cells without any treatment). From Figure 7d, the compound-**1b**-treated cells exhibited higher relative fluorescence intensity than of positive-control-treated cells, and the same can also be visualized from the cells in Figure 7b,c. Green fluorescence is observed in the cells that are activated through the release of caspase-3 proteins, and, at the same time, the cells are alive and undergoing the apoptotic pathway because of the effect of compound **1b**. The observation of high fluorescence levels for the compound-**1b**-treated cancer cells confirms the active role played by the caspases (in support of caspase-3 activity). However, no significant fluorescence is observed in the untreated controls (Figure 7a) because of the absence of caspase activity, while slight fluorescence in Mel (Figure 7b) and significant fluorescence in compound-**1b**-treated cells (Figure 7c) confirm the caspase-3 activity, along with the apoptosis mechanism in live cells.

Figure 6. Comparison of flow cytometric analysis results for ROS generation in HepG2 cancer cells at 48 h incubation. From the figure, A, B, and C correspond to the negative control (without any treatment), the positive control (Mel; 15 µg/mL), and compound 1b (at IC₅₀), respectively, while 1, 2, and 3 stand for the cell population tested, number of cells exhibiting H₂DCFDA fluorescence, and % of fluorescent intensity, respectively.

Figure 7. Caspase 3 activity comparison of HepG2 cancer cells, followed by the treatment of IC₅₀ concentration of compound 1b (c) with that of positive control of Mel (15 µg/mL) (b) and negative control (a) over a 48 h period. Relative fluorescence intensity of all the samples is compared in (d).

In the present study, the anticancer activity of spirooxindole–pyrrolidine heterocyclic hybrids has been investigated against cancer cells, where the results have proven that all the derivatives exhibit some level of activity. This activity seems to increase with that of concentration and incubation period, and the highly active compound was found to be **1b**, with the lowest IC₅₀ value of ~24 µg/mL over a treatment time of 48 h. Further testing of the intracellular pathways followed by compound **1b** treatment proved that the cancer cells are experiencing the apoptotic mechanism, with the release of oxidative stress mediated by ROS and the involvement of caspases. Comparing the present study results with that of our recently published work [31], it primarily indicates that the spirooxindole–pyrrolidine heterocycles are a little less active than the *N*-pyridinylmethyl-engrafted bisarylmethylidenepyridinones. The lower activity is reflected in the form of IC₅₀ values, i.e., we observed lower activity at about 12 µg/mL (24 h) for the *N*-pyridinylmethyl-engrafted bisarylmethylidenepyridinones and at 24 µg/mL (48 h) for the spirooxindole–pyrrolidine heterocycles. However, the other assay results, such as apoptosis, oxidative stress, and caspase activity, were almost the same for both of the chemical moieties under similar experimental conditions. In addition, the response of the spirooxindole–pyrrolidine heterocycles towards noncancer cells is the same as the *N*-pyridinylmethyl-engrafted bisarylmethylidenepyridinones, revealing that the chemical moiety is able to differentiate between the aggressively growing cancer and nonaggressive healthy normal cells. This shows that the spirooxindole–pyrrolidine heterocycles can be employed for the prolonged therapeutic actions against cancer cells; for a similar but quicker effect, the *N*-pyridinylmethyl-engrafted bisarylmethylidenepyridinones are more suitable.

4. Conclusions

The biological evaluation of our synthesized compounds **1(a–h)** revealed that all the derivatives exhibit anticancer activity against HepG2 cancer cells, and this activity is quite high at the 48 h incubation period. Additionally, the mechanistic investigation of the highly active compound **1b** provided the information that the cells are experiencing an apoptotic pathway, with the induction of oxidative stress carried by ROS and the involvement of caspases. The observed activity of compound **1b** at a very low IC₅₀ value and, at the same time, its not-very-aggressive nature against noncancer cells indicate the potential role that can be played by spiropyrrolidine derivatives as chemotherapeutic agents.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2227-9717/8/11/1473/s1>.

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