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Cytotoxic potential of di-spirooxindolo/acenaphthoquinone andrographolide derivatives against MCF7 cell lines

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Di-spiro andrographolide derivatives have been prepared from isatin/acenaphthoquinone, N-benzyl glycine and andrographolide via azomethine ylide cycloaddition reaction. Cytotoxic effect of the synthesized molecules has been studied against MCF-7 breast cancer cell lines. The compounds induced apoptotic cell death as revealed by increased labeling with Annexin-V, decreased polarization of cell mitochondria, and increased reactive oxygen species production. Using FACS and western blot analysis, the compounds were observed to block the cell cycle at S and G2-M phases by causing DNA damage that halted replication and ultimately caused apoptosis. Activation of caspases 7 and 9 suggested that caspase pathways were involved in inducing apoptosis.

Introduction

The human race has hugely benefited from drugs discovered from different natural sources, especially plants. Recent studies conducted by the World Health Organization (WHO) have brought out the fact that about 80% of the world’s population rely on traditional medicine.\textsuperscript{1} At present about 121 drugs prescribed in USA trace their origin to natural resources, 90 of which either directly or indirectly come from plant kingdoms.\textsuperscript{2} Between the years 1981-2006, about a hundred anticancer agents have been developed, of which twenty five are natural product derivatives, eighteen are natural product mimics, eleven candidates are derived from a natural product pharmacophore, and nine are pure natural products.\textsuperscript{3}

Breast cancer is a very common cancer in women both in the developed and less developed world and is the second leading cause of cancer death in women. Worldwide over 508,000 women are estimated to have died in 2011 due to breast cancer.\textsuperscript{4} Although breast cancer is thought to be a disease of the developed world, almost 50% of breast cancer cases and 58% of deaths occur in less developed countries.\textsuperscript{5} It is therefore of immense interest to search for new drug candidates from the plant kingdom to tackle this menace.

The herb Andrographis paniculata Nees (Acanthaceae) is popular in India, China and other Asian countries due to its different uses in traditional medicine. The metabolites isolated from this herb are mainly diterpenoids, flavonoids and sterols. The major constituent is andrographolide (1), a labdane diterpene. Extracts of this herb and its various constituents are reported to have a broad range of biological activities.\textsuperscript{6-10} In the recent past, potent anti-cancer activities of andrographolide have been described.\textsuperscript{10-13}

However, despite its impressive biological activities; the major drawback of andrographolide is poor water solubility making it difficult to prepare formulations for clinical use. So, various semi-synthetic analogues are being developed and evaluated in order to find out a better lead.\textsuperscript{14-18} Jada et al. recently reported that 14-acetylandrographolide is more potent in vitro against leukemia compared to andrographolide.\textsuperscript{15,16} Another report identified DRF 3188, a novel derivative of andrographolide having α,β-unsaturated ester side chain at C14, to have better anti-cancer activity than the parent molecule.\textsuperscript{17} Encouraged by these reports, we prepared a few dispiro analogues of andrographolide via azomethine ylide cycloaddition.\textsuperscript{19} The results of the biological evaluation of these compounds\textsuperscript{20} encouraged us further to build a library of dispiro analogues containing both spiro-oxindole and pyrrolidine/pyrrolizidine rings attached to andrographolide.\textsuperscript{21} Preliminary bio-evaluation indicated the promise held out by N-benzyl glycine derivatives that needed to be further explored. In the present study, we evaluated the anticancer efficacy of some of these analogues of andrographolide which showed good MTT value for MCF-7 breast cancer cell line. As apoptosis is the physiologically desired pathway of cell death by the anticancer agents\textsuperscript{22,23} we wanted to explore the involvement of apoptosis in cell death induced by the andrographolide derivatives.

Results and discussion

Chemistry

As described earlier that the dispiro products were derived from Andrographolide (1, isolated from A. paniculata in the usual way),\textsuperscript{24,25} isatin derivatives (2 a-k) or acenaphthoquinone (3), and N-benzyl glycine via azomethine ylide cycloaddition\textsuperscript{19} which were subsequently crystallized from acetonitrile-benzene (Scheme 1) and characterized by spectral analysis.\textsuperscript{21}
Biological studies

Previously the cytotoxic efficacies of different di-spiropyrdindino and dispiropyridinidoxindole analogues prepared by the cycloadition of azomethine ylides of sarcosine or proline (described herein as sarcosine and proline series respectively) at 12,13 double bond of andrographolide were evaluated by our group. The results encouraged us to build a library of similar dispiro derivatives by using N-benzyl glycine as amino acid component and carry out their biological evaluation. The present communication describes the anticancer role of these analogues, five of which displayed good MTT values against MCF-7 breast cancer cell line.

Cytotoxicity of andrographolide derivatives (N-benzyl glycine series) on cancer cell lines

Cell proliferation inhibition was tested by MTT assay following treatment of cells with 12 derivatives (N-benzyl glycine series) and their cytotoxic potential was tested in MCF 7 (breast carcinoma), HepG-2 (hepatocellular carcinoma), HeLa (cervical carcinoma), A431 (epidermoid carcinoma), and CHO (Chinese hamster ovary) cell lines. Out of the 12 derivatives, compounds 5b, 5c, 5g, 5i, and 8 were found to be the most potent in different cancer cell lines. These compounds showed very promising activity particularly in MCF7 cell line, having IC50 values of 10.9±0.4, 12.7±0.1, 12.8±0.9, 13.7±1.7 and 8.9±1.6 µM respectively after 72 h incubation. Based on the results, these compounds have been chosen for subsequent studies in MCF7 cells. DMSO (0.1%), representative of the highest concentration used (100µg/ml), showed no effect on cell viability, confirming its biological inertness.

Compounds 5(b, c, i, g) and 8 induce morphological changes in MCF-7 cell

It is desirable that cell death induced by an anticancer agent occurs through apoptosis. Light microscopic study of the cells treated with IC50 concentrations of the compounds for 24 h indeed revealed characteristic apoptotic changes like progressive detachment and rounded cells. This study indicates that 5b, c, i, g, and 8 induced morphological changes in MCF-7 cells (see supplementary fig S1).

The test compounds cause externalization of phosphatidylserine

Annexin V FITC and PI double-labeling studies were used for the detection of phosphatidylserine externalization. Flow cytometric analysis showed that MCF7 cells treated with test compounds showed higher number of annexin V positive cells than control (Figure-1). The percentage of viable cells was significantly low for all the samples. The total numbers of annexin V binding in MCF7 cells detectable for 5(b, c, i, g) and 8 exposures are 20.3, 27.2, 23.1, 37.7 and 43.2% respectively after 24 h, and 20.0, 91.9, 61.9, 98.3, and 99.6% respectively after 48 h. The percentage of PI positive cells for these compounds (upper left quadrant) at baseline were minimal and remained comparable at 24 and 48 h, being 0.5, 0.9, 0.6, 0.9, and 0.3% in the former case, and 2.2, 0.2, 1.4, 0.0, and 0.0% in the latter, implying negligible occurrence of necrosis. Taken all together, these compounds cause externalization of phosphatidyl serine, indicative of apoptosis.

Figure-1: Detection of apoptosis in MCF-7 cell line. (a) Induction of apoptosis by 5b, 5c, 5g, 5i, and 8. Cells were treated for 24 and 48 h with indicated concentrations of the compounds and were co-stained with PI and Annexin-V FITC prior to analysis using flow cytometry as described in materials and methods.

The compounds induce the mitochondrial pathway of apoptosis

The mitochondrial membrane potential was measured by staining with JC-1, a lipophilic cationic fluorescent dye capable of selectively entering mitochondria and acting as a dual emission probe that reversibly changes colour from red to green in concert with polarization of mitochondrial membrane. Since oxygen consumption is a surrogate indicator of mitochondrial bioenergetics, depleted cellular respiration coupled with increased ROS production may lead to mitochondrial...
depolarization.\(^2^7\) A gradual time dependent change of red to green fluorescence was observed in MCF7 cells after treatment with the test samples (Figure 2). The green fluorescence in control cells was 10.0%, which increased to 79.0% for 5b, 62.3% for 5c, 50.0% for 5i, 46.1% for 5g, and 83.0% for 8 at 24 h of treatment and to 88.9% for 5b, 89.8% for 5c, 97.5 for 5i, 93.1% for 5g, and 96.4% for 8 at 48 h. This was also reflected in the altered ratio of green/blue fluorescence which in control cells was 9.0. Following the addition of 5(b, c, i, g) and 8, this ratio demonstrated a time-dependent decline (respectively 0.266, 0.605, 1.002, 1.173, and 0.205 at 24 h and 0.126, 0.114, 0.026, 0.069, and 0.037 at 48 h) suggesting the occurrence of depolarization of mitochondria by these compounds.

ROS induces DNA damage which plays role in apoptosis induced by the compounds

ROS plays an important role in the induction of apoptosis in various types of cells. Mitochondria are the major site of ROS production in mammalian cells, and superoxide \(\text{O}_2^-\) appears to be the primary ROS produced as the result of single electron reduction of \(\text{O}_2\).\(^{2^8,2^9}\) However, excessive ROS generation beyond a certain threshold level renders cancer cells highly susceptible to chemotherapy induced death. Under such circumstances, ROS are known to function as the initial mediators of apoptosis.\(^3^0\) To examine whether 5(b, c, i, g) and 8 affect the oxidative function of the cell, we quantified ROS at different time points by measuring the fluorescent signals of the products using FACS. Cells were treated with IC\(_{50}\) concentrations of the samples for 24 h (Figure 3a). The result showed significant accumulation of ROS in this period. A shift to the right (red) generally indicates the ROS accumulation. Figure 3a shows the percentage increase caused by the compounds compared with control in different time periods. The observed increase in fluorescence was attributable to the ability of the compounds to generate ROS in MCF-7 cells.

**Figure-2: Detection of mitochondrial membrane potential.** MCF-7 cells were incubated with IC\(_{50}\) concentrations of 5b, 5c, 5i, 5g and 8 for 24 or 48 h. Cells were loaded with mitochondrial sensor dye JC-1 as described in materials and methods. Change in membrane potential was detected by the shift from red to green fluorescence in time dependent manner.

The compounds induce S phase cell cycle arrest in MCF 7 cells

Apoptosis occurs through cell cycle arrest at a specific phase of cell division. The percentage increase or decrease in the number of cells in each phase of the cell cycle compared to that in the control was determined and represented by DNA histogram. As summarised in Figure 4, treatment with apoptosis doses of these...
compounds for 24 and 48 h caused a gradual increase in the number of cells in S phase. The increases in S and G2-M populations at 24 h of treated MCF-7 cells (S and G2-M in Fig. 4) were respectively 12.2%, 4.9% for 5b, 11.3%, 5.3% for 5c, 14.8%, 2.0% (decrease) for 5i, 12.2%, 6.9% for 5g and 7.6%, 5.6% for 8 in comparison with vehicle control. At 48 h, the increase in S population was respectively 25.4% for 5b, 5c 19.5%, 5i 31.8%, 5g 26.7% and 22.3% for 8. In each case there was a concomitant reduction in the number of cells in the G0-G1 (G0-G1 in Fig. 4) phase at 24 and 48 h in comparison with vehicle control (respectively 17.1%, 21.6% for 5b, 16.8%, 16.6% for 5c, 12.9%, 26.2%) for 5i, (19.2%, 25.3%) for 5g and (13.2%, 27.2% for 8). This experiment revealed that the compounds induced a dramatic increase in arrest cells at S phase at the two time points.

**Figure-4:** Study of cell cycle arrest in MCF-7 cells. DNA histograms show the effect of treatment of 5b, 5c, 5i, 5g and 8 on cell-cycle of MCF-7 cells. The cells were treated for 24 and 48 h with indicated concentrations of the compounds. The area of peaks corresponds to DNA content in different phases of cell cycle.

**Discussion**

In our previous studies, we evaluated the cytotoxic efficacy of 15 different di-spiropyrrulidino and dispiropyrrolizidino oxindole andrographolide analogues prepared by cycloaddition of azomethine ylide and sarcosine or proline at 12,13 double bond of andrographolide. The tests performed using HCT116 and five other carcinoma cell lines, viz. MiaPaCa-2, HepG2, HeLa, A375 and A549, revealed that three compounds showed better results than andrographolide itself. The results encouraged to build a library of dispiro analogues containing both spiro-oxindole and pyrrolidine/pyrrolizidine rings attached to andrographolide for further biological evaluation. The findings indicate the promise held out by N-benzyl glycine derivatives as five out of 12 compounds show very promising activity against five cancer cell lines. Of these, 5b, 5c, 5g and 5i carry CH3, Cl, F or Br group in the aromatic ring. But the acenaphthoquinone ring containing derivative (8) gave the best result.

We tested the effects of these compounds in five different cancer cell lines (Figure 2A), viz. MCF7 (breast carcinoma), CHO (Chinese hamster ovary carcinoma), HepG-2 (hepatocellular carcinoma), A431 (epidermoid carcinoma), and HeLa (cervical carcinoma). Interestingly, all the compounds are relatively more active against MCF-7 breast cancer cell line (Figure 2B). An important feature of these lead derivatives is that cell death triggered by them involves apoptosis. Typical features of apoptosis like cell rounding, nuclear condensation and DNA fragmentation, and externalization of phosphatidyl serine were observed in response to treatment with 5(b, c, i, g) and 8.

Apoptosis induced by these derivatives were found to be mediated via mitochondrial pathway with loss of mitochondrial membrane potential, activation of caspases 9, cleavage of PARP and increase of cytosolic cytochrome c level. They also induced ROS mediated apoptotic cell death which was evidenced by flow cytometric assay. The data suggests that the mode of action of the derivatives on MCF-7 cells is similar to that of andrographolide. Therefore these compounds may be regarded as potential anticancer candidates.

From the above study it appears that three of this N-benzyl glycine series of andrographolide derivatives (5c, 5g and 5i) possess better activity compared to serine/proline series. It also shows that chlorinated, fluorinated and brominated derivatives are in general more potent than or iodinated derivatives, which may be related to the electronegativity and size of the substituents. But the only acenaphthoquinone derivative (8) tested appears most potent among all the derivatives.

Bromine is larger in size and also moderate halogen bond acceptor than fluorine. The C-Br bond is stable enough, allowing its insertion on diverse heterocycles of pharmacological value.

Another feature of brominated drugs concerns its binding affinity for some cellular proteins. Replacing hydrogen by bromine also provides a substantial alteration on the size and shape issues. The subunits bearing bromine can be accommodated in tight and deep cavities, as well as in hydrophobic pockets of the biological targets.

Chlorine occupy an intermediate position in between fluorine and bromine. It is a better halogen bond acceptor, besides being smaller in size than bromine. The C-Cl bond is also stable enough, allowing its insertion on diverse heterocycles of pharmacological value similar to bromine.

From our study, three halogenated andrographolide derivatives have been identified to show more potent cytotoxic activity than andrographolide. Halogens are important functional groups. But the number of halogenated anticancer drugs is less compared to the derivatives having other functional groups. Binary protein halogenated ligand complexes are more stable than non-halogenated ligands because of favorable electrostatic interactions of the halogen bonds. Cyclin-dependent kinase 2 (CDK2) plays critical roles in important intracellular pathways.
and cell cycle progression, being thus an attractive biological target for drug development. Auffinger and co-workers showed that halogenated ligands bind to the cyclin-dependent kinase 2 (CDK2), leading to more stable complexes than non-halogenated ligands. The incorporation of halogen atoms into new bioactive chemical entity is commonly used to increase membrane permeability and thereby improve oral absorption. Halogenation also enhances the blood brain barrier (BBB) permeability and this is a pre-requisite for many drugs. In this respect our study of new halogenated anticancer agents are important findings and adds a new approach in cancer research. It is believed that carbon-halogen bonds are not easily metabolized by the cytochrome p450 system and, therefore, it is a feasible strategy to block the metabolically labile positions of a particular new bioactive chemical entity, providing an explanation for our results.

Conclusion

In conclusion, our study demonstrates that five andrographolide derivatives show more potent anticancer activity than andrographolide itself. In this respect, our findings are important to explore anticancer agent(s). Probably, biological potency of the derivatives results from the combined effect of the nature of halogens and the structures of the compounds to which they are attached. In addition, we can conclude that the potent compound 8, which is structurally different from other analogues to some extent, may be similar to andrographolide in its effect towards apoptosis induction in cancer cells.

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Notes and Reference