Selective Cytotoxic Activities of Two Novel Synthetic Drugs on Human Breast Carcinoma MCF-7 Cells

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Abstract. *Background: Breast cancer is the second leading cause of cancer deaths in US women. We evaluated two novel compounds, piperidinyl-diethylstilbestrol (DES) and pyrrolidinyl-diethylstilbestrol (DES) for cytotoxicity against brine shrimp larvae, MCF-7 and rat normal liver cells. Materials and Methods: In vivo cytotoxicity was evaluated against shrimp larvae for 24 h, while in vitro cell toxicity was evaluated by dye binding crystal-violet method after 48 h. The role of these compounds on different phases of the cell cycle was assessed by flow cytometry. Results: In shrimp assay, piperidinyl-DES and pyrrolidinyl-DES were potent with 50% effective dose (ED_{50}) values of 7.9±0.38 and 15.6±1.3 μM, respectively. In MCF-7 and normal liver cells, the 50% lethal concentration (LC_{50}) values were 19.7±0.95, 17.6±0.4 μM and 35.1 and >50 μM, respectively. Cell cycle analyses indicated that MCF-7 cells were arrested at the G_0/G_1 stage with these compounds. Conclusion: The results indicate that pyrrolidinyl-DES possesses highly selective, potent anticancer activity.*

Although extensive preclinical and clinical studies have resulted in modest success in decreasing the morbidity of breast cancer, to date no successful chemotherapeutic drug is available to control this disease. Every year, several thousands of new chemical entities undergo screening in various cell cultures but eventually do not achieve drug status due to severe side-effects. Ideally, one of the criteria for a drug being good is that it should not exhibit any undesirable side-effects on normal cells. In an effort to minimize the side- effects, the National Cancer Institute and others (1-10) have been exploring the vast resource of natural products for potent and selective anticancer agents but as yet, the success rate in discover such molecules has remained very low. Our earlier attempts to purify new compounds from plant sources have resulted in two novel anticancer molecules (8). One of these molecules was reported to exhibit selective anticancer activity in various cell cultures (6) but the subsequent preclinical studies with this compound in tumor-bearing nude mice did not yield good results due to its solubility problem (unpublished observations). As part of our ongoing drug discovery research in synthesizing potent and selective anticancer synthetic compounds, we identified two novel drugs, piperidinyl-DES and pyrrolidinyl-DES, and screened initially for lethality against brine shrimp larvae and extended the evaluations for cytotoxicity as well as antiproliferative potential by flow cytometry in the human estrogen-dependent MCF-7 breast carcinoma cell line. In addition, the cytotoxicity of these compounds was also evaluated in a normal rat liver cell line, CRL-1439, for comparison.

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Materials and Methods

Chemicals. Trypsin, minimum essential medium, antibiotic-antimycotic solution (Ab/Am) and 4-hydroxy tamoxifen were purchased from Sigma Chemical Company (St. Louis, MO, USA). Fetal bovine serum, RPMI-1640 (modified), penicillin, streptomycin and L-glutamine were purchased from Media Tech (Herndon, VA, USA). Cosmic calf serum was purchased from Hyclone Lab Inc (Logan, UT, USA) and gentamicin reagent solution from Invitrogen (Carlsbad, CA, USA). Human insulin (Novolin R) was purchased locally. All other routine chemicals were of analytical grade.

In vivo lethality test by brine shrimp bioassay. In this technique, the *in vivo* potency of piperidinyl-DES and pyrrolidinyl-DES was evaluated against shrimp larvae. The assay was performed in triplicate vials at different doses (5, 10 and 15 μM) as described by Meyer *et al.* (11) and modified by McLaughlin (12). Shrimp larvae in ethanol (0.15%, v/v) in artificial seawater served as control. 4-Hydroxy tamoxifen (5, 10 and 15 μM) was used as a positive control. At the end of the incubation period, the surviving larvae per dose were counted and utilized for analysis.

Table I. *Comparison of cytotoxic activities of synthetic compounds. For in vivo study, the brine shrimp larvae were treated with test compounds at 5, 10 and 15 μM for 24 h, and the live shrimps were counted and analyzed. The ED_{50} values were calculated from the plots. Each value represents the mean±SEM (n=3). For in vitro study, the cells were treated at 5, 10, 25, 50, 75 and 100 μM for 48 h and evaluated for cytotoxicity by the crystal violet method. Each value represents the mean±SEM (n=8-12).*

	Piperidinyl-DES	Pyrrolidinyl-DES	4-Hydroxy tamoxifen
In vivo, ED_{50} (μM±SEM)			
Brine shrimp assay	7.9*±0.38 (3.2 μg/ml)	15.6±1.3 (6.3 μg/ml)	9.2±0.7 (3.5 μg/ml)
In vitro, LC_{50} (μM±SEM)			
CRL-1439 normal liver cell line	35.1±0.77 (14.6 μg/ml)	>50 (>20 μg/ml)	20.25±0.25 (7.8 μg/ml)
MCF-7 adenocarcinoma cell line	19.7[a#]±0.95 (8.2 μg/ml)	17.6[a]±0.4 (7.1 μg/ml)	15.6[a]±1.0 (6.0 μg/ml)
Selectivity index	1.78	>2.84	1.29

*Highly significant in comparison to pyrrolidinyl-DES in shrimp assay ($p<0.01$, n=3); ainsignificant in comparison to pyrrolidinyl-DES or tamoxifen ($p>0.05$, n=8); #highly significant in comparison to normal cells ($p<0.01$, n=8-12), one-way ANOVA, Bonferronic multiple comparison test.

Cell cultures and maintenance. Human estrogen-dependent breast carcinoma (MCF-7) cell line and normal rat liver cell line (CRL-1439) were purchased from the American Type Culture Collection (Rockville, MD, USA) and were used in the present investigation. These cell lines were maintained as described elsewhere (6, 13).

Treatments with drugs. The cytotoxic studies with cell cultures were carried out in polystyrene, flat-bottom 96-well microtiter plates as described elsewhere (9). All test compounds were dissolved in ethanol at 10 mM stocks, from which working stocks were prepared in complete culture medium prior to treatments. Cells were treated with piperidinyl-DES or pyrrolidinyl-DES at different concentrations (5 to 100 μM). 4-Hydroxy tamoxifen (5 to 100 μM) was used as a positive control. The culture plates were incubated for 48 h continuously without further renewal of growth media in 5% CO_2 at 37˚C, with the plates capped in the normal fashion. All studies were repeated at least twice (n=8 to 12). After 48 h of exposure, the cytotoxicity of the compounds was evaluated by dye uptake assay using crystal violet as described elsewhere (2).

Cell cycle analysis by flow cytometry. MCF-7 cells (0.18×10^6/ml) were treated with ethanol (0.15% , control), or piperidinyl-DES (5, 10 and 15 μM), or pyrrolidinyl-DES (5, 10 and 15 μM), or 4-hydroxy tamoxifen (5 μM) in triplicate dishes for 48 h in a 5% CO_2 incubator at 37˚C. Cells were fixed in 100% ice-cold ethanol for at least 24 h at 4˚C. They were then stained with 1 ml of freshly prepared staining solution containing 50 μg/ml propidium iodide, 1.3 mg/ml ribonuclease A and 1 mg/ml D-glucose and incubated at room temperature for 1 h in the dark with occasional stirring. The proportion of cells in each stage was assayed within 2 h by using a FACSCalibur flow cytometer (Becton Dickerson, San Jose, CA, USA). In each sample, a total of 13,000 individual events from the gated subpopulation were analyzed separately. CellQuest software was used for acquisition and analysis of the data, and the percentage of cells in each phase was determined.

Selectivity index (SI). In the present study, the degree of selectivity of the synthetic compounds is expressed as per the previous report (14) with a minor modification (6): SI=LC_{50} of pure compound in a normal cell line/LC_{50} of the same pure compound in cancer cell line, where LC_{50} is the concentration required to kill 50% of the cell population.

Statistical analysis. The experimental results are presented as mean±standard error of the mean (SEM). The data were analyzed for significance by one-way ANOVA and then compared by Bonferronic multiple comparison tests using GraphPad Prism Software, Version 3.00 (San Diego, CA, USA). The LC_{50} and effective dose needed to kill 50% of shrimp larvae (ED_{50}) were determined from the line graphs where both curves crossed (15). The test value of $p<0.01$ was considered highly significant.

Results

Toxicity of synthetic compounds to shrimp larvae. Addition of piperidinyl-DES or pyrrolidinyl-DES (or 4-hydroxy tamoxifen) at 5, 10 or 15 μM to sea salt solution did not result in any precipitation. This is one of the primary requirements for an ideal compound for preclinical studies. It was observed that both piperidinyl-DES and pyrrolidinyl-DES caused significant ($p<0.01$) death of larvae at concentrations greater than 5 μM in a dose- dependent manner in comparison to the untreated control. Towards the end of incubation, the shrimp larvae in control vials were seen to be swimming actively, while the others in treated vials appeared very weak in terms of their swimming abilities. The ED_{50} values of piperidinyl-DES, pyrrolidinyl-DES and 4-hydroxy tamoxifen were (±SEM) 7.9±0.38 (3.2 μg/ml), 15.6±1.3 (6.3 μg/ml) and 9.2±0.7 μM (3.5 μg/ml), respectively (Table I).

Cytotoxicity of synthetic compounds to cell cultures. Based on the high activity against the shrimp larvae, the *in vitro* anticancer activity of both test compounds was evaluated at different concentrations against human MCF-7 breast adenocarcinoma cells after 48 h of treatment. It was observed that in comparison to the control, both compounds caused significant ($p<0.01$) dose-dependent cell death to a similar extent. The LC_{50} values (±SEM) for piperidinyl-DES and pyrrolidinyl-DES were 19.7±0.95 μM (8.2 μg/ml) and

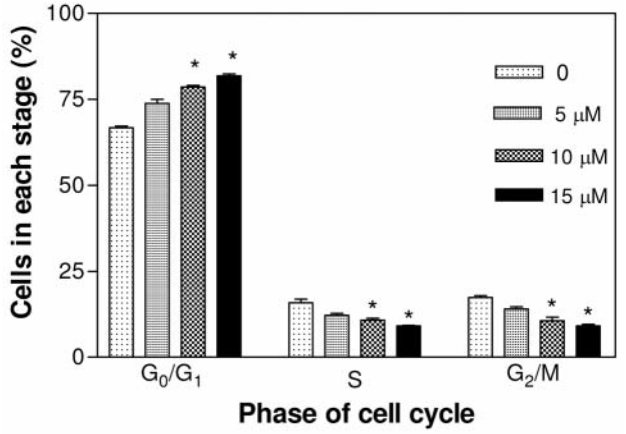

Figure 1. *Effect of piperidinyl-DES on MCF-7 cell cycle. The cells at a starting density of 0.18×10⁶/ml per dish in complete medium containing 10% regular FBS were treated with 0, 5, 10 or 15 μM of the compound for 48 h. Cells were harvested and stained by propidium iodide staining solution for 1 h in the dark and analyzed by flow cytometry. Data are represented as mean±SEM (n=3, *p<0.01; highly significant in comparison to control; one-way ANOVA, Bonferronic multiple comparison test).*

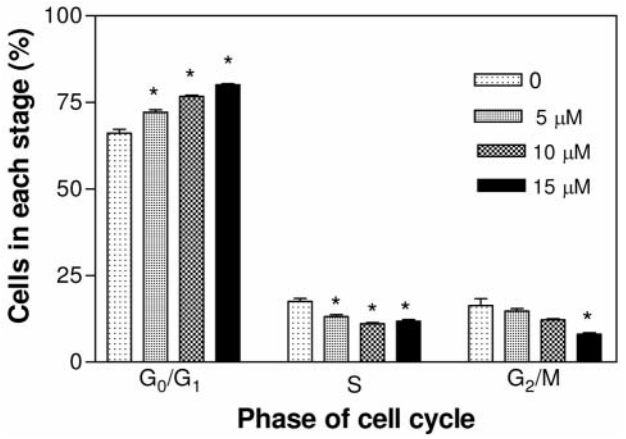

Figure 2. *Effect of pyrrolidinyl-DES on MCF-7 cell cycle. The cells at a starting density of 0.18×10⁶/ml per dish in complete medium containing 10% regular FBS were treated with 0, 5, 10 or 15 μM of the compound for 48 h. Cells were harvested and stained by propidium iodide staining solution for 1 h in the dark and analyzed by flow cytometry. Data are represented as mean±SEM (n=3, *p<0.01; highly significant in comparison to control; one-way ANOVA, Bonferronic multiple comparison test).*

17.6±0.4 μM (7.1 μg/ml), respectively (Table I). 4-Hydroxy tamoxifen under our experimental conditions had an LC_{50} of 15.6±1.0 μM (6 μg/ml) on MCF-7 cells (Table I), but others (16) reported a much higher value (31 μM) for the same cell line. The reason for this could be due to differences in the assay methods (13).

When tested in a rat normal liver cell line, CRL-1439, both compounds exhibited significant differential cytotoxic activities (Table I). For example, the LC_{50} of piperidinyl-DES was 35.1±0.77 μM (14.6 μg/ml), while in the case of pyrrolidinyl-DES, it was >50 μM (>20 μg/ml). The LC_{50} of 4-hydroxy tamoxifen in the normal liver cells was 20.25±0.25 μM (7.8 μg/ml).

Inhibition at G_0/G_1-phase of the MCF-7 cell cycle. Figures 1 and 2 show the effects of increasing concentrations of piperidinyl-DES and pyrrolidinyl-DES on MCF-7 cell progression through G_0/G_1-, S- and G_2/M-phases. It was observed that in comparison to the control, piperidinyl-DES and pyrrolidinyl-DES at different concentrations for 48 h resulted in cell cycle arrest significantly ($p<0.01$) in the G_0/G_1-phase and in the reduction of the number of cells in the S- and G_2/M-phases. This demonstrates that both these synthetic compounds arrest cell cycle progression at the G_0/G_1 to S transition. The accumulation of cells at this phase was significant at 10 and 15 μM with both compounds ($p<0.01$), correlating with a subsequent significant decrease in S- and G_2/M-phase cells at these concentrations ($p<0.01$). The positive control 4-hydroxy tamoxifen arrested the cells at the G0/G1-phase of the cell cycle.

Discussion

The *in vivo* brine shrimp lethality is a simple bioassay considered as a useful tool for primary screening of various kinds of water-soluble compounds (2-7, 9, 11, 12). The significant difference (2-fold) in the ED_{50} values of both synthetic compounds here may suggest that the mode of action of these compounds in brine shrimp larvae were entirely different. Comparison of ED_{50} values (Table I) clearly shows that piperidinyl-DES was approximately 2-fold more toxic to shrimp larvae in comparison to pyrrolidinyl-DES ($p<0.01$). Based on the observation that the ED_{50} values of these compounds were less than 1,000 μg/ml, both were considered very active as per the earlier standard for potency of compounds in shrimp assay (11).

In the case of our *in vitro* studies with MCF-7 cells, the similarity of the LC_{50} values of piperidinyl-DES, pyrrolidinyl-DES and 4-hydroxy tamoxifen suggests that they may have the same target sites in these cells namely the estrogen receptor. This speculation, however, needs further studies for confirmation.

The significant difference in LC_{50} values of piperidinyl-DES and pyrrolidinyl-DES in the normal cell line reflects an interesting difference in the target sites in adenocarcinoma and normal cells. As per the earlier standard (17), pure compounds with potency of 4 μg/ml or less in cell culture studies are further considered for evaluation as chemotherapeutic agents in preclinical studies using animal models. In our study, in spite of slightly higher LC_{50} values of piperidinyl-DES and pyrrolidinyl-DES in MCF-7 cells in

comparison to the earlier set standard for potency (17), the differential toxicity exhibited by these compounds is highly significant and may warrant further active investigation.

As the SI demonstrates the differential activity of a pure compound, the greater the SI value is, the more selective it is. An SI value less than 2 indicates general toxicity of the pure compound (14). Based on this, the SI data shown in Table I indicate that pyrrolidinyl-DES exhibits a high degree of cytotoxic selectivity. It is, however, unclear, as to why this compound exhibited a similar extent of toxicity in brine shrimp and MCF-7 cells. Interestingly, the SI for 4-hydroxy tamoxifen, which is widely used as an antiestrogen treatment for human breast cancer, was less than 2. This suggests its general toxicity to cells, as per the earlier standard (14).

Comparison of brine shrimp and cell culture assays (Table I) reveals that piperidinyl-DES shows a 2-fold differential toxicity in shrimp larvae as well as in MCF-7 cells, while this was not observed in the case of pyrrolidinyl-DES. The high degree of correlation between the cytotoxicity of the shrimp larvae and MCF-7 in this study is consistent with previous reports (7, 18) with different anticancer agents.

Conclusion

One of the important criteria for a therapeutic drug for cancer is to have minimum or no side-effects on normal body cells of patients undergoing chemotherapy. One way to achieve this is by employing lower doses of drugs. This invariably implies that the drug should not only have high potent activity at lower concentrations but also should exhibit high degree of selectivity. The present *in vitro* studies demonstrate the ability of the synthetic compound pyrrolidinyl-DES for high selective toxicity at lower concentrations (Table I). Since estrogens are associated with the etiology of human breast cancer, inhibition of estrogen-dependent cell growth by synthetic compounds such as pyrrolidinyl-DES, shown by cell cycle analysis in this study (Figures 1 and 2), may become a good therapeutic strategy to use as an antagonist for treatment of this dreaded disease. Further studies, however, are required to ascertain the antiestrogenic activity of pyrrolidinyl-DES. Current studies are under way to determine the efficacy of this compound as an anticancer activity in animal models.

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