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We have generated novel analogs of progesterone (PgA) as potent inhibitors of the MDR1 multidrug resistant phenotype in breast cancer. The objectives of this Research Project include the optimization of the PgA's MDR1-reversing activity through the generation of additional compounds, using an analog design approach, the definition of these compounds' *in vivo* efficacy, and of their mechanism of action. In the course of the fourth year of the Project, we have: 1. tested the *in vivo* MDR-reversing activity of PgA4, the most potent among our initial progesterone analogs; 2. designed and synthesized additional progesterone analogs and evaluated their structure-activity relationships; 3. related the *in vitro* activity of some of the analogs to their *in vitro* toxicity.

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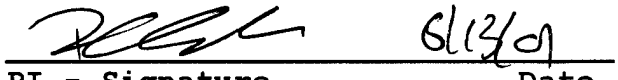

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INTRODUCTION

Resistance to available chemotherapy is critical to our failure to cure advanced breast cancer. The mechanisms responsible for drug resistance in breast cancer are likely to be multiple. However, expression of one of them, the protein product of the MDR1 gene P-glycoprotein (Pgp), appears to correlate with a more than 3-fold increase in the relative risk of breast cancers to fail to respond to systemic chemotherapy [1]. Pgp is a membrane glycoprotein whose expression *in vitro* confers a multidrug resistant phenotype, apparently by an active efflux mechanism from the cell membrane bilayer [2]. Pgp substrates include several critical anticancer agents including anthracyclines, taxanes, vinca alkaloids and epipodophyllotoxins [3]. Inhibition of the resistance to anticancer substrates conferred by Pgp has been demonstrated for several compounds [4], several of which may act by a competitive mechanism [5-12]. Only, relatively few of these agents have so far reached clinical trial [13]. These include "first generation" MDR1-reversing drugs originally designed for different purposes (e.g., verapamil and cyclosporin A), whose efficacy is often limited by toxicity related to their original pharmacological purpose [14-17]. "Second generation" MDR1 inhibitors include drugs structurally related to first generation drugs, but selected to be less toxic. These include dexverapamil, dexniguldipine and S9788, drugs whose use is anyway limited by cardiovascular toxicity [18-21]. Finally, "third generation" drugs are supposedly the result of a targeted drug discovery approach. Perhaps, the most advanced in clinical trial is the cyclosporin analog SDZ PSC 833 (Valspodar). Evidence of activity of this drug has been obtained in refractory or relapsed multiple myeloma and acute myelogenous leukemia [22, 23]. However, SDZ PS 833 administration can cause hyperbilirubinemia (frequently) and, sporadically, severe ataxia [23, 24]. The toxicities observed so far highlight the need for more rationally designed agents with improved therapeutic index.

We have used a rational analog-based approach to the design of new and more effective MDR1-reversing agents. We have selected a natural and relatively non-toxic steroid, progesterone, as our lead compound, and introduced modifications partly based on the available knowledge about the structural determinants of both MDR1 and of the steroid hormonal activity. Progesterone is the most potent of the physiological steroids for reversing the MDR1 phenotype [25]. Bulky substitutions in the C7 position of the steroid nucleus inhibit its hormonal activity [26]. Moreover, bulky substitutions also appear to increase MDR1-reversing activity, as further confirmed by tests of the activity of C11-substituted steroidal agents carried out in this lab (data are shown in the original Proposal). Using these clues, we designed and synthesized progesterone analogs where a bulky side chain, including one or two aromatic rings and a urea group, is substituted on the C7 position of the steroidal nucleus. These novel analogs of progesterone (PgA) showed not only an increased MDR1 reversing activity (up to 35 fold higher than the parental compound, in terms of both chemosensitization and increased cell accumulation of vinblastine), but also decreased progesterone agonist and glucocorticoid agonist/antagonist activities. The most favorable ratio of MDR1-reversing to hormonal activity was observed with PgA4, an analog where the C7 side-chain includes two aromatic rings (rings E and F) connected by a urea-containing bridge (data presented in the original Proposal).

BODY OF REPORT

METHODS

Cell lines. For our *in vitro* and *in vivo* experiments we used cells transduced with a retroviral vector directing the constitutive expression of the Pgp gene (MDA435/LCC6^{MDR1}) and their parental, Pgp-negative MDA435/LCC6 human breast cancer cells. Both MDA435/LCC6 and MDA435/LCC6^{MDR1} cells are estrogen and progesterone receptor negative, grow as monolayer cultures *in vitro*, and as rapidly proliferating solid tumors and malignant ascites *in vivo* in nude mice. The cells were routinely grown *in vitro* in Improved Minimal Essential Media (Biofluids) containing 5% fetal bovine serum in a 5% CO_2: 95% air atmosphere.

Doxorubicin accumulation assay: The MDR1-reversing activity of all new agents was evaluated in terms of their effect on doxorubicin accumulation in MDA435/LCC6^{MDR1} human breast cancer cells [27]. Pgp-negative MDA435/LCC6 [27] were used as a negative control and to evaluate non-specific effects. MDA435/LCC6 and MDA435/LCC6^{MDR1} cells were plated at 2.5 x 10^5 cells/well in the wells of 24-well plates, and incubated for 24 hrs at 37 °C in a humidified, 95% air/5% CO_2 atmosphere. 24 hours after plating, cells were treated by exchanging spent media with the media containing the test compounds at 4 different concentrations + doxorubicin 4 μM (0.5 ml/well). All treatments were carried out in triplicate. Cell cultures were then reincubated at 37 °C for 3 hours. Treatments were stopped by carefully washing wells once with 0.5 ml/well ice-cold NaCl (0.15 M). Cells from reference wells in each plate were counted. Doxorubicin was extracted from the cell monolayer in the remaining wells by first adding 0.75 ml dH_2O, and then 0.75 ml 40% trichloroacetic acid per well. Plates were incubated overnight at 4 °C in the dark. For spectrofluorimetry, 1.2 ml of the extract from each well were transferred into 13 x 100 mm borosilicate glass tubes placed in the 10 x 10 rack of a Hitachi A3000 Autosampler. The autosampler was connected to a Hitachi F4500 Spectrofluorimeter. Fluorescence of each sample was read at 500 nm excitation and 580 nm emission wavelengths. The doxorubicin concentration in each sample was calculated by interpolation on a doxorubicin standard curve and normalized by extract volume and number of cells per well.

Analysis of data from accumulation studies. Results were plotted both in terms of the estimated drug concentration per 10^6 cells and as the percentage of drug accumulation differential (difference between accumulation in untreated MDA435/LCC6 and MDA435/LCC6^{MDR1} cells) reversal *vs.* test compound concentration. MDR1-reversing potency was calculated in terms of the chemosensitizer concentration that induces a 50% reduction in the drug accumulation differential (EC_{50}) in the Pgp-positive cells, by interpolation on the dose-response curve. A "MDR1-specific" EC_{50} value was obtained by interpolation on the dose0response curves corrected by subtraction of the accumulation effect in the Pgp-negative cells.

Evaluation of *in vitro* toxicity. MDA435/LCC6 and MDA435/LCC6^{MDR1} cells were plated in 96-well plates and, 24 hours later, were exposed to growth media containing different

concentrations of the test agents (progesterone, C7 progesterone analogs, cyclosporin A or verapamil) for 5 days. Cell cultures were then fixed and stained by incubation in a 0.5% (w/v) crystal violet solution in 25% methanol (v/v). After plates had dried, the dye was extracted in 0.1 M sodium citrate in 25% methanol (v/v) and absorbance was read at 540 nm using a microplate spectrophotometer. Absorbance directly correlates with cell number in this assay. Cell survival curves were obtained by plotting absorbance values (as % of untreated controls) against drug concentration. The toxicity of each drug was summarized in terms of IC_{50}, the concentration decreasing cell density by 50% at the end of the treatment period. For those drugs that produced a detectable IC_{50}, the ratio of IC_{50} values in MDA435/LCC6^{MDR1} and MDA435/ LCC6 cells provided an estimate of the relative resistance of Pgp-positive cells. Ratios of > 1 are suggestive of a possible transport of the test drugs by Pgp.

Evaluation of *in vivo* toxicity (preliminary).

Before we could proceed to test the *in vivo* MDR1-reversing activity of PgA4, we needed to confirm the lack of toxicity of the PgA4 treatment regimen to be used in combination with doxorubicin. NCr *nu/nu* female athymic nude mice (two per treatment group) were treated with either PgA4, prepared at 1.5 mg/ml in 20% hydroxypropylcyclodextrin, 15 mg/Kg, or the same amount of vehicle *sc*, twice a day for 3 days. Mice were observed for immediate or delayed signs of toxicity (mortality, altered behavior, decrease in body weight gain).

Evaluation of *in vivo* MDR1 reversing activity.

NCr *nu/nu* female athymic nude mice were inoculated *i.p* with either $1x10^6$ MDA435/LCC6 (control) or MDA435/LCC6^{MDR1} cells. Treatment was started as soon as the ascites started to develop. The mice were assigned to 4 treatment groups according to the whether the treatment included PgA4 and/or doxorubicin. PgA4, 1.5 mg/ml in 20% hydroxypropylcyclodextrin, was administered *s.c.* at 0.010 ml/g body weight (15 mg/Kg) twice a day for 3 days.. Control groups were treated with an equivalent amount of vehicle. Doxorubicin, 12 mg/Kg, was administered as a single *iv* inoculation immediately following the 5th inoculation of PgA4. Control mice received an equivalent amount of saline solution. 24 hours after doxorubicin inoculation, the mice were sacrificed. The ascitic fluid was collected and spun in a microfuge at 4000 rpm for 4 min, the relative pellet extracted with 20% trichloroacetic acid and the extract evaluated for doxorubicin content fluorimetrically, as described above for *in vitro* evaluations.

CHEMISTRY

General Considerations

All reactions were carried out under an atmosphere of nitrogen using standard Schlenk techniques [28]. Benzene and chloroform were distilled from CaH_2, stored over 3Å molecular sieves and deaerated by purging with nitrogen immediately before use. Thin-layer chromatography was performed using Merck glass plates pre-coated with F_{254} silica gel 60; compounds were visualized by UV and/or with *p*-anisaldehyde stain solution. Flash chromatography was performed using EM Science silica gel 60, following the procedure of Still [29], with the solvent mixtures indicated. Melting points were measured on a Thomas-Hoover Capillary Melting Point Apparatus, and are uncorrected.

Reagents

All reagents were purchased from commercial suppliers, and used as received, unless indicated otherwise. Dioxane was purchased from Aldrich in Sure-Seal bottles.

Spectroscopic Methods

NMR spectra were measured on Nicolet NT 270 and Varian Mercury 300 MHz instruments at the Georgetown NMR Facility; chemical shifts are reported in units of parts per million relative to Me_4Si. All spectra are recorded in $CDCl_3$. Significant ^1H NMR data are tabulated in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), coupling constants in Hertz, and number of protons. ^{13}C NMR spectra were recorded at frequencies of 67.9 and 75.6 MHz. IR spectra were measured on a MIDAC Corp. or a Mattson Galaxy 2020 Series FTIR, as neat films; absorption bands are reported in cm^{-1}. Low- resolution mass spectra were measured on a Fisons Instruments MD 800 quadrupole mass spectrometer, with 70 ev electron ionization, and a GC 8000 Series gas chromatograph inlet, using a J & W Scientific DB-5MS column of 15 m length, 0.25 mm i.d. and 0.25 μm film thickness. Mass spectra data are given as m/e, with the relative peak height following in parentheses.

Compound Characterization

All new compounds were characterized by ^1H NMR, IR and ^{13}C NMR spectroscopies. Fast atom bombardment mass spectra (FABMS) were recorded at the University of Maryland College Park of Mass Spectrometry Facility. Literature references are given for all known compounds, with the exception of those that are commercially available; all known compounds were identified by ^1H NMR spectroscopy.

Preparation and Characterization of Compounds (Scheme 1)

Step 1: Synthesis of Dehydroprogesterone. *p*-Toluenesulfonic acid monohydrate (11.0 g, 63.9 mmol) was dehydrated in freshly distilled benzene (320 mL) via azeotropic refluxing employing a Dean-Stark trap. After 1 h, cooled the solution for 0.5 h, and progesterone (5.0

g, 15.9 mmol) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (4.6 g, 20.3 mmol) were added. The olive mixture was refluxed for 3 hrs, and then was filtered through a pad of Celite. The filtrate was washed with sat. NaCl (5 x 20 mL), followed by 1% NaOH solution until it gave clear solution, and then dried over $MgSO_4$. The solvent was removed under reduced pressure and purified by chromatography.

Step 2: Synthesis of 7 -[4'-(aminophenyl)thio]-pregna-4-ene-3,20-dione (PgA1), 7 -[4'-(aminophenyl)thio]-pregna-4-ene-3,20-dione (PgA37) or 7 -[4'-(aminophenyl)thio]-pregna-4-ene-3,20-dione (PgA39). Dehydroprogesterone (1.65 g, 5.28 mmol), NaOH (pellet, 116 mg, 2.9 mmol), and 4-aminothiophenol (for PgA1), or 3-aminothiophenol (for PgA37) or 2-aminothiophenol (for PgA39; each, 1.32 g, 10.56 mmol) were placed in a Schlenk tube, which was purged with a constant flow of N_2 (g). Deoxygenated anhydrous dioxane (25 mL) was added and heated at 74°C for 6 days. The mixture was then concentrated under reduced pressure, purified by chromatography.

Step 3: Synthesis of additional progesterone analogs. A suspension of PgA1, or PgA37, or PgA39, in degassed chloroform was treated with the appropriate isocyanates under N_2. The mixture was stirred for 12 hrs, and then chromatographed directly on silica gel to afford the corresponding ureas as oil. The resulting oil was stirred in ether until white powder came out.

RESULTS

OBJECTIVE 1:

EVALUATION OF PGA4'S *IN VIVO* MDR1 REVERSING ACTIVITY

We evaluated the ability of PgA4 to increase the cellular accumulation of doxorubicin specifically in MDA435/LCC6^{MDR1} ascites cells. A first experiment was carried on a limited number of mice per treatment group. PgA4 was administered *s.c.* at the dose of 13 mg/Kg every 12 hours for 3 days. Doxorubicin was administered *i.v.* at 12 mg/Kg at the time of PgA4's 5th inoculation. The ascites were collected 24 hours after doxorubicin treatment and handled as already described. Doxorubicin concentration was evaluated fluorimetrically in both the ascites cell pellet and the relative supernatant. The results are reported in Fig. 1 in terms of ratio of intracellular/extracellular doxorubicin concentration. The results confirm the lower ratio of cellular/extracellular concentrations in LCC6^{MDR1} as compared to LCC6 ascites (these results are statistically significant). More interestingly, the data also show that PgA4 treatment increases the ratio specifically in Pgp(+) LCC6^{MDR1} ascites. So, these data represent the first evidence suggesting that PgA4 is an effective *in vivo* MDR1-reversing agent. However, because of the low number of mice in this pilot study, the effect of PgA4 on LCC6^{MDR1} ascites did not reach statistical significance. Consequently, the experiment needed to be repeated with a larger number of animals per treatment group.

In a second experiment, in order to increase the number of animals per treatment group, 60

NCr *nu/nu* mice were all inoculated with MDA/LCC6^{MDR1} cells. 20 animals were treated with doxorubicin (12 mg/Kg i.v.) and 20 more with doxorubicin +PgA4 (30 mg/Kg per inoculation). Two 10-mice control groups were treated respectively with vehicle only and PgA4 only. The results of this test are represented in Fig. 2. Unfortunately, the effect of PgA4 on doxorubicin accumulation in MDA435/LCC6^{MDR1} ascites cells is minimal and does not allow to confirm the *in vivo* MDR1-reversing efficacy that the prior test had suggested.

Possible reasons for the failure of the doxorubicin accumulation model to validate PgA4's *in vivo* MDR1-reversing activity:

1. Though we consistently used the same strain of animals for our *in vivo* doxorubicin accumulation assays, it is still possible that doxorubicin pharmacokinetics may vary in mice at different times for the different experiments (though all from the same breeder). So, the 24-hour time between doxorubicin treatment and ascites collection (based on the result of preliminary evaluations in the first years of this project) may not have been optimal in all the studies. A possible solution would be to sacrifice mice from each treatment group at different times following doxorubicin treatment: this would allow to compare the "Area Under the Curve" (AUC) for intracellular drug rather than drug accumulation at one single fixed time. This solution would require additional personnel to be assigned to the task.
2. PgA4 may not be effective because it does not reach the target at effective concentrations for metabolic and/or pharmacokinetic reasons. This possibility may be addressed by: defining PgA4's pharmacokinetics, metabolism and target concentration; increasing the potency of PgA4; increasing PgA4's resistance to metabolic degradation.

Future directions

A final verification of the *in vivo* MDR1-reversing activity of our tests compounds will be obtained by standard tumor growth delay (on solid tumor models) and/or % ILS (Increased Life Span, using ascites models).

OBJECTIVES 2 AND 3: BRIDGE AND F-RING OPTIMIZATION, *IN VITRO* MDR1-REVERSING ACTIVITY AND TOXICITY

MDR1-REVERSING ACTIVITY

The *in vitro* MDR1-reversing activity of our test compounds and of the reference standard agents verapamil and cyclosporin was evaluated in terms of effect on the intracellular concentration of doxorubicin in MDR1-expressing human breast cancer cells (MDA435/LCC6^{MDR1}). Potency was quantified in terms of EC_{50}, the drug concentration necessary to reduce by 50% the difference in doxorubicin intracellular accumulation between MDA435/LCC6^{MDR1} and the parental MDR1-negative MDA435/LCC6 cells. The results are reported in Tables 1a, 1b and 1c in terms of potency relative to the parental compound progesterone without (second column from the right) or with correction (first column from the right, labeled "MDR1-specific") for the non-specific effect of the test compound on the MDR1-negative control cells.

We have designed and synthesized additional C7 progesterone derivatives with the aim of further optimizing these compounds' MDR1-reversing activity and characterizing their structure-activity relationships. Overall, we have so far synthesized and tested the in vitro MDR1-reversing activity of 24 progesterone analogs. Their structure and MDR1 reversing activity (in terms of potency relative to the parental compound progesterone) are summarized in Fig. 3 and Tables 1a, 1b and 1c .

Length of the alkyl chain distal to the C7 urea-E ring moiety. The C7 progesterone analog PgA3 (7α[4'-(N-ethylaminoacylaminophenyl)thio]pregna-4-ene-3, 20-dione), which includes in the C7 side chain an ethyl group bound to an aromatic e-ring through a urea group, is about 40-fold more potent than the parental compound progesterone in reversing the MDR1 phenotype (in terms of effect on doxorubicin accumulation in MDA435/LCC6^{MDR1} cells). The length of the alkyl group ("ethyl" in PgA3) may effect the compound's MDR1activity. A propyl group (as in PgA41) in lieu of the ethyl group (as in PgA3) may somewhat increase the activity. However, further elongation of the alkyl chain bound to the urea group appear to be counterproductive, possibly because of problems of steric hindrance: butyl- and hexyl- substituents (as, respectively, in PgA36 and PgA35) appear to decrease the activity with respect to PgA3. However, the data for PgA35, PgA36, and PgA41 need confirmation.

Role of the urea group. An obvious way to evaluate the role of the urea group would be to evaluate the activity of of a C7 progesterone analog deprived of this group. In PgA1 (7α[4'-(aminophenyl) thio] pregna-4-ene-3, 20-dione), the precursor of most of our C7 analogues, a primary amine group substitutes the alkylurea group in the *para* position of the E-ring. Unfortunately, the MDR1 activity of PgA1could not be evaluated because of problems with the compound's solubility. We were, however able to compare the activity of PgA37, an isomer of PgA1 where the amine group is substituted in *meta* as compared to *para* position, with its ethyl urea derivative PgA38 (isomer of PgA3). The ethylurea derivative appears to be about twice as potent as the parental compound. The comparison, however, is not perfectly adequate, because the role of the urea group cannot be discriminated from the role of the distal group length.

Position of the alkylurea substituent on the E ring. Comparison of PgA3 (alkylurea group in *para* position on the E-ring), PgA38 (*meta*) and PgA40 (*ortho*), suggests slightly higher activities when the alkylurea group is substituted in the *meta* position (about 60-fold more potent than progesterone) on the E-ring. A loss of potency was observed for the *ortho* isomer (about 18-fold more potent than progesterone). Advantage conferred by the addition of an aromatic F-ring (PgA13) is lost when this is part of group substituted in the *meta* position of the E-ring.

Role of polarity in the distal C7 side chain. Polarization of the ethyl substituent in PgA3 by chlorination (PgA2) does not appear to obviously alter its ability to modulate doxorubicin accumulation in MDR1 cells.

Role of F-ring and bridge length. The presence of an aromatic F ring (as in PgA13) confers more than a 3-fold increase in MDR1 cell-specific doxorubicin accumulation effect (as compared to PgA3), but only when the ring is directly attached to the urea group. Longer bridges between E and F ring fail to show an obvious (>2-fold) advantage when compared with the effect of compounds without a F ring. This evidence may suggest that the aromatic F ring in PgA13 is more favorably located for $\pi-\pi$ interaction with the aromatic amino acids on Pgp.

Role of a partial positive or negative charge on the F ring. The increased potency conferred by an aromatic F ring (in PgA13), appears to be lost following its substitution with an electron-withdrawing p-trifluoromethyl- group (PgA28), suggesting that acquisition of a partial positive charge negatively affects the ability of the F ring to interact with Pgp. Also the addition of other electron-withdrawing or electron-donating groups on the F ring (as in the PgA13 analogs PgA20, PgA30, PgA31, PgA32, and PgA34 appears to decrease the MDR1 reversing effect. However, a simple steric hindrance effect of the substituent on the F ring cannot be completely ruled out.

Role of a third aromatic ring ("G"). Addition of a third aromatic ring, distal to the "F" ring, as in PgA29, appears to inhibit the MDR1 reversing potency, possibly because of steric hindrance.

IN VITRO ACTIVITY/TOXICITY RATIO

We have evaluated the *in vitro* toxicity of some of our early test compounds and compared it with that of the reference MDR1 reversing agents verapamil and cyclosporin A. The cytotoxicity of the different compounds is reported in Table 2 in terms of IC_{50}, the concentration inhibiting 50% cell growth. For a more adequate comparison of drug efficacies, table also provides an estimate of "toxicity-corrected" efficacy, in terms of the ratio of IC_{50} (for toxicity) and EC_{50} (for MDR1 reversing activity) values for each drug. For PgA3 and PgA4, a 50% level of growth inhibition could not be reached at the maximum concentration that, for limits of solubility, could be obtained *in vitro*. So, for these compounds the reported ratios represent only minimum estimates. The results show that, while the MDR1-reversing potency of PgA3 and PgA4 is about 4-times higher than that of verapamil and comparable to that of cyclosporin A, the toxicity-corrected *in vitro* MDR1 reversing efficacy of these compounds far exceeds cyclosporin A's, an agent with a high level of *in vitro* toxicity.

By showing a relative resistance of MDR1-expressing cells to PgA2 and PgA5 (about 2-3 fold), the results of the *in vitro* toxicity tests also suggest that these C7 progesterone analogs are themselves substrates for Pgp. Consequently, it appears likely that these progesterone analogs inhibit the multidrug resistant phenotype by competing with the anticancer drugs for Pgp's transport mechanism.

KEY RESEARCH ACCOMPLISHMENTS

- Initial evaluation of one of the Progesterone analogues *in vivo* MDR1 reversing activity

- Optimization of *in vitro* MDR1-reversing activity: the most potent analogue (PgA13) is now more than 150-fold more potent than the parental compound progesterone, more than 15-fold more potent than verapamil, and about 3-fold more potent than cyclosporin A).

- Characterization of the *in vitro* pharmacological activity/toxicity ratio of some of the analogues: this ratio is at least 20 times better for the progesterone analogue PgA4 than for the standard reference drug cyclosporin A.

- Evaluation of structure-activity relationships of the C7-moiety of C7-progesterone analogues: length, role of a second and third aromatic ring, polarity, position of side chain on E-ring.

REPORTABLE OUTCOMES

Abstracts

- Leonessa, F., Kim, J.-H., and Clarke, R. Structure-activity relationships of MDR1 reversal by novel progesterone analogs. "*Molecular Determinants of Sensitivity to Antitumor Agents: an AACR Special Conference in Cancer Research*", Whistler, British Columbia, Canada, March 4-8, 1999
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- Lu, L., Leonessa, F., Clarke, R., and Wainer, I.W. Frontal chromatographic analysis of drugs interactions with immobilized P-glycoprotein. *Proceedings of the 1999 AACR-NCI-EORTC International Conference (#516), Washington, D.C., November 16-19, 1999*
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Patents

- Clarke, R., Talebian, A., Ghiorghis, A., Leonessa, F., Hammer, C: Progesterone Analogues to Reverse Multidrug Resistance (U.S. Patent 60/000,440, World Patent 08/667,542)

CONCLUSIONS

Using a substrate (doxorubicin) accumulation endpoint, we have tested the *in vivo* MDR1 reversing activity of PgA4, the most potent *in vitro* MDR1 inhibitor among our early compounds. Though the results of an initial test were suggestive of an *in vivo* effect, differences were not statistically significant. Also a second *in vivo* test failed to demonstrate a statistically significant effect. In future experiments, we will further test the *in vivo* activity of PgA4, using alternative endpoints (solid tumor growth, survival), and cell models (P388/ADR cells).

An expanded panel of C7-progesterone analogs has allowed us to further investigate the relation between the MDR1 reversing activity and the structure of our C7-progestone analogs. The structural features that we have considered include: the length of the C7 moiety (distal to the urea group), the presence of a urea group, the position of the alkylurea substituent on the E ring, the polarity in the distal C7 side chain, the presence of a second and third aromatic ring in the C7 moiety, polarity of the F ring and length of the bridge between rings, and hydrophobicity. Our best compound (PgA13) is more than 150-fold potent than the parental compound progesterone, 10-fold more potent than verapamil and almost 3-fold more potent than cyclosporin.

Not only are some of the C7-progesterone analogs equally or more potent than the classical MDR1 reversing cyclosporin A but, at least *in vitro*, some of then appear much less toxic (20-fold or more) at concentrations which are equiactive on the MDR1 phenotype.

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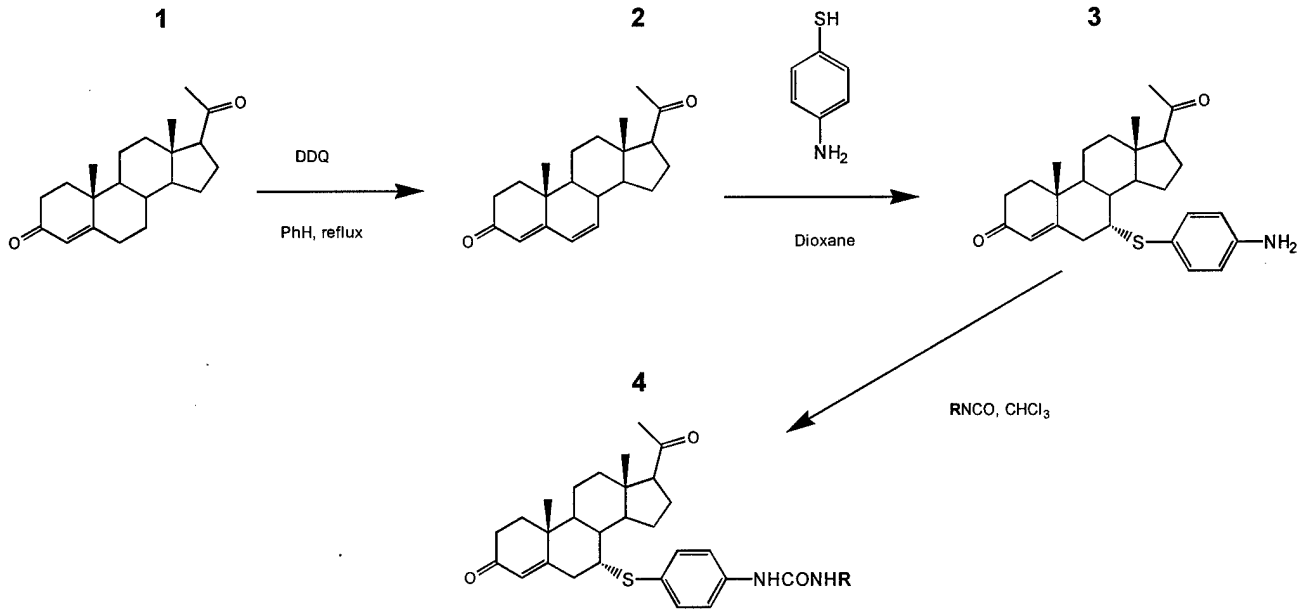
APPENDICES

Scheme 1. Synthesis of PgA compounds



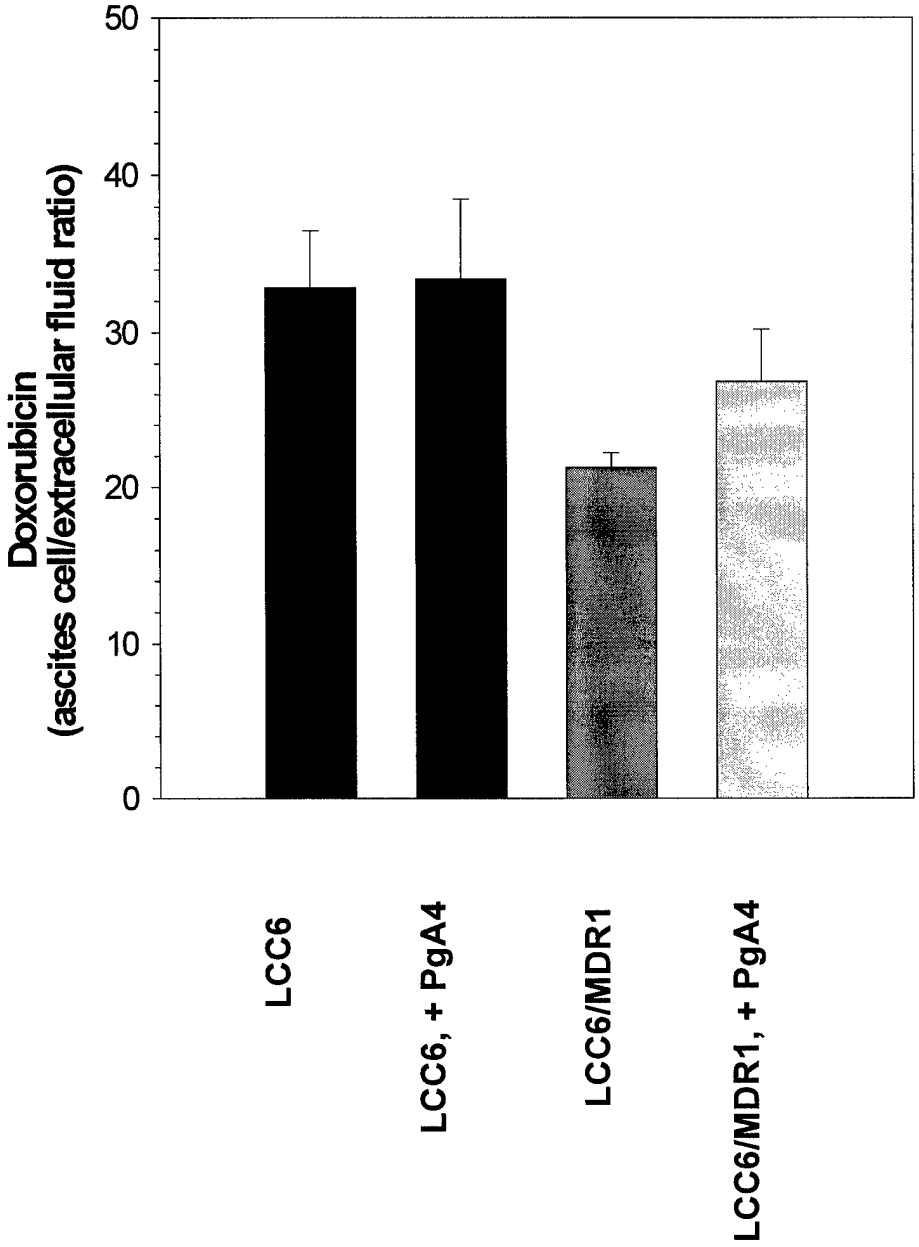


FIGURE 1. *In vivo* effect of PgA4 treatment on the ratio of the intracellular/ extracellular concentration of doxorubicin in MDA435/LCC6 and MDA435/LCC6^{MDR1} ascites cells.

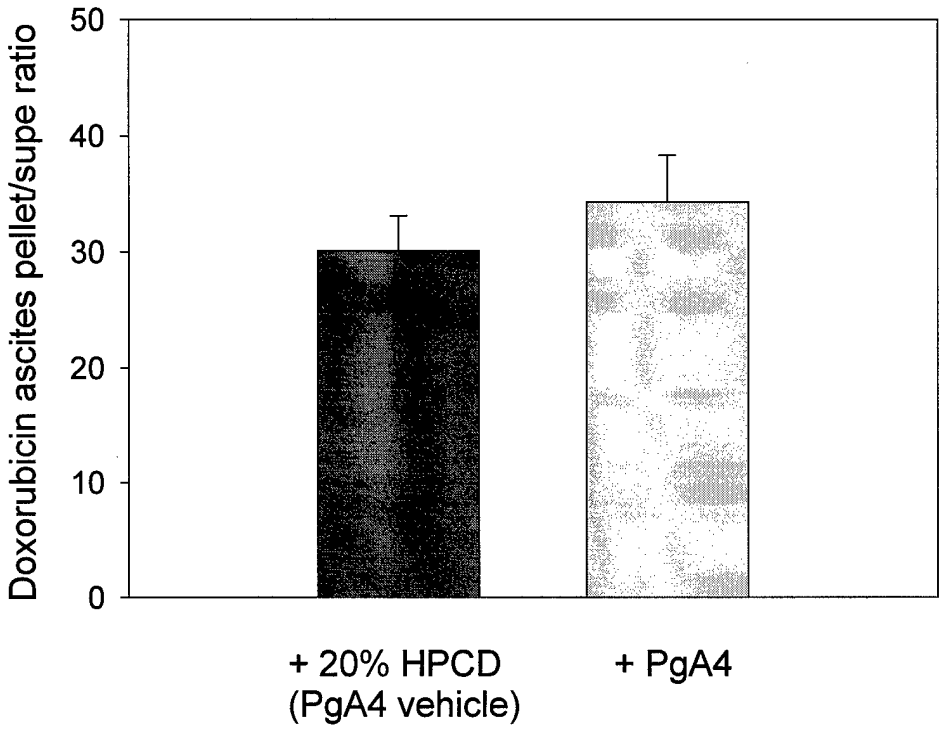
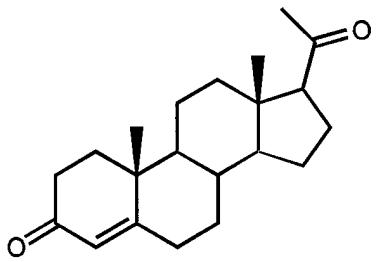
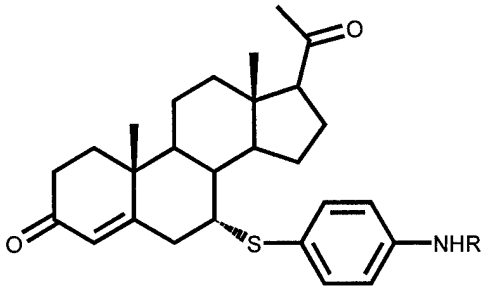


FIGURE 2. *In vivo* effect of PgA4 treatment on the ratio of the intracellular/ extracellular concentration of doxorubicin in LCC6/MDR1 ascites cells

Progesterone



PgA: Series I



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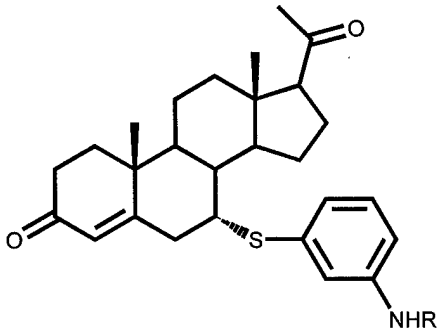

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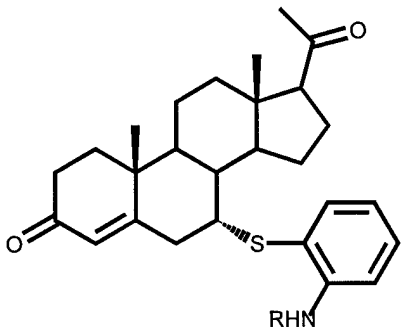
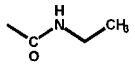
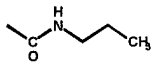
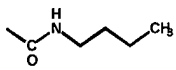
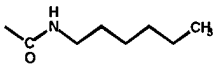
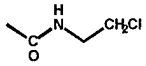
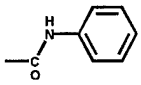
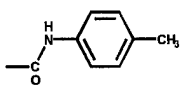
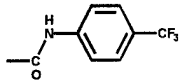


FIGURE 3. Structure of progesterone and of its C7 analogues (see also Table1)

TABLE 1

Relative potency of C7 progesterone analogs in reversing the MDR1 phenotype:

PgA: Series I

Analog	*"R" Function*	*MDR1-reversing potency, relative to progesterone*	
		LCC6/MDR1 cells	*MDR1-specific*
PgA3		*31.3*	*42.7*
PgA41		*61.6*	*66.9*
PgA36		*17.1*	*15.5*
PgA35		*26.7*	*27.1*
PgA2		*40.5*	*60.2*
PgA13		*104.8*	*166.7*
PgA20		*90.1*	*108.0*
PgA28		*26.8*	*40.2*

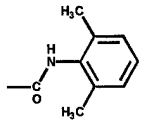
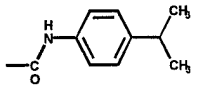
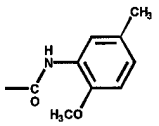
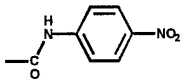
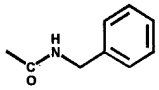
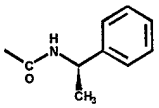
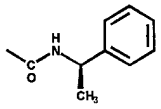
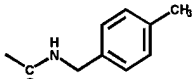
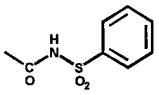
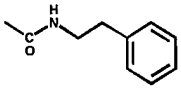
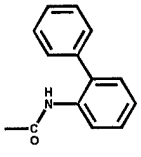
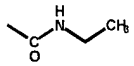
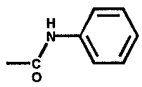
PgA30		*24.2*	*25.6*
PgA31		*22.6*	*31.1*
PgA32		*42.9*	*47.9*
PgA34		*30.6*	*39.1*
PgA8		*35.3*	*40.1*
PgA4(+)		*37.2*	*44.8*
PgA4(-)		*28.3*	*23.8*
PgA11		*32.8*	*30.5*
PgA5		*1.5*	*1.1*
PgA12		*49.2*	*50.8*
PgA29		*8.5*	*16.4*

TABLE 1/B

Relative potency of different C7 progesterone analogues in reversing the MDR1 phenotype

PgA: Series II

Analog	*"R" Function*	*Potency, relative to progesterone*	
		LCC6/MDR1 cells	*MDR1-specific*
PgA37	*H*	*27.8*	*29.5*
PgA38		*54.5*	*69.4*
PgA43		*63.4*	*67.6*

PgA: Series III

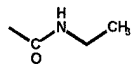
Analog	*"R" Function*	*Potency, relative to progesterone*	
		LCC6/MDR1 cells	*MDR1-specific*
PgA39	*H*	*20.4*	*16.9*
PgA40		*16.7*	*17.7*

TABLE 1/C

MDR1 REVERSING ACTIVITY OF REFERENCE STANDARD MDR1-REVERSING AGENTS

Analog	*"R" Function*	*Potency, relative to progesterone*	
		LCC6/MDR1 cells	*MDR1-specific*
Cyclosporin A		*41.9*	*60.6*
Verapamil		*9.2*	*10.2*

TABLE 2

In vitro toxicity and MDR1-activity / toxicity ratio of C7-progesterone analogs

Drug	*IC_{50} in MDR1- cells (μM)*	*IC_{50} in MDR1+ cells (μM)*	*Relative resistance in MDR1+ cells*	*IC_{50}/EC_{50}**
Progesterone	*35.35*	*44.69*	*1.26*	*1.1*
PgA2	*3.33*	*9.77*	*2.93*	*13.9*
PgA3	*> 20*	*> 20*	*--*	*> 20.0*
PgA4	*> 20*	*> 20*	*--*	*> 21.2*
PgA5	*20.45*	*38.32*	*1.87*	*1.0*
Cyclosporin A	*0.49*	*0.85*	*1.73*	*1.2*
Verapamil	*65.81*	*62.20*	*0.95*	*15.1*

** IC50 in MDR1-positive cells / MDR1-specific EC_{50}*