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We have generated novel analogs of progesterone (PgA) as more 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 more compounds by analog design; definition of their *in vivo* efficacy and of the mechanism of MDR1-reversal. In the course of the second year of the Project, we have: *1.* Completed the characterization of a model for the preliminary evaluation of *in vivo* MDR1-reversing activity (based on the doxorubicin accumulation in MDR1-positive ascites cells), defined PgA4's formulation for *in vivo* treatment and tested its *in vivo* toxicity; *2.* generated novel PgA structures by stepwise analog design, obtaining data for initial structure-activity considerations and a further 3-fold increase in MDR1-reversing potency; *3.* generated and started the characterization of a novel affinity chromatography column (with the MDR1 product, P-glycoprotein, immobilized in the solid phase) to test the competitive nature of PgA-induced MDR1 reversal .

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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 increased relative risk of breast cancers failing 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].

The number and heterogeneity of MDR1 substrates is matched by the variety of non-anticancer compounds that are able to inhibit the MDR1 multidrug resistant phenotype [4]. Binding to, and transport by, Pgp have been demonstrated for several of these compounds [5-9]. This, and evidence from more direct binding studies [10-12], suggest that several compounds may inhibit the MDR1 phenotype by a competitive mechanism.

Relatively few of the many MDR1-reversing agents active *in vitro* have so far reached clinical trial [13]. Most "first generation" drugs were originally designed for different purposes; their use for MDR1-reversal is limited by toxicity that is often related to their original pharmacological purpose. For example, racemic verapamil is active on Pgp at concentrations that are difficult to reach at the plasma level without significant cardiovascular toxicity [14]. Progesterone and tamoxifen dose-limiting toxicities are represented by central nervous system side effects [15, 16]. Cyclosporin, a drug for which evidence of clinical efficacy has been obtained in myeloma patients [17], is an immunosuppressive agent and can cause hyperbilirubinemia at the doses required for MDR1 reversal [13]. "Second generation" MDR1 inhibitors include drugs selected to be less toxic. These include dexverapamil, dexniguldipine and S9788. Nonetheless, clinical use of all three drugs is 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 has caused 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 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) show 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).

This Research Project has the following objectives (as defined in the Statement of Work): (1) *in vivo* evaluation of PgA4's toxicity and potency for reversal; (2) bridge optimization and *in vitro* potency; (3) F-ring optimization and *in vitro* potency; (4) *in vivo* evaluation of bridge/F-ring optimized analogues; (5) mechanistic studies.

During the first two years, we have actively pursued our first three Objectives. In the first year, we have: (1) carried out a meta-analysis of published studies to demonstrate the impact of Pgp expression on breast cancer response to chemotherapy; (2) started to define a formulation based on the use of hydroxypropylcyclodextrin to optimize PgA4's delivery (PgA4 is poorly soluble in aqueous solution and this represents a potential limitation to its use *in vivo*); (3) tested the possible use of doxorubicin accumulation in solid tumors as a preliminary endpoint of *in vivo* MDR1-reversing activity.

In the following sections, we describe the work accomplished in the second year of this research project. We have further characterized the *in vivo* differential accumulation of doxorubicin in Pgp-positive and –negative human breast cancer cells grown as ascites. We have confirmed PgA4's formulation for *in vivo* treatment and initiated preliminary evaluations of PgA4's *in vivo* toxicity and MDR1-reversing activity. New analogs have been synthesized, allowing both an optimization of MDR1-reversing activity *in vitro* (now, almost 100-fold higher than progesterone for the most potent compound) and an improved understanding of PgA's structure-activity relationships. Finally, we have started the characterization and optimization of a new chromatographic system, based on the use of Pgp-containing columns, which will allow us to pursue Objective 5 (Mechanistic studies) in the course of the third year of this Project.

In the Results and Discussion section our data are discussed and the next steps dscribed, in relation to the stated objectives of this research project. Objectives 2 and 3 (respectively, Bridge and F ring optimization) represent different aspects of a study to optimize structure-activity relationships and optimize *in vitro* and *in vivo* MDR1-reversing activity. Data concerning these two objectives are consequently presented and discussed under a common subsection.

BODY OF REPORT

METHODS

Evaluation of UV profile and Specific Absorption Coefficient (E_1^1)

PgA4 was dissolved in methanol at 1 mg/ml. An aliquot from this stock was diluted to 10 µg/ml in methanol. The UV profile of the diluted solution was evaluated using a Beckman DU-460 spectrophotometer by scanning its absorbance between 190 and 390 nm wavelength. Based on PgA4 UV profile, we chose 245 nm as our reference wavelength for future evaluations, as this corresponds to a plateau in the UV profile. The specific absorption coefficient E_1^1, defined as the absorption at path length 1 cm and compound concentration 10 mg/ml, was calculated by multiplying the recorded absorption at 245 nm wavelength by 1000. The average PgA4's E_1^1 was 376.1 +/- 4.3 (mean +/- SE of 3 separate determinations).

Evaluation of PgA4 solubility in different vehicles.

A saturating amount of PgA4 was suspended in the test vehicle and kept shaking overnight, at room temperature. The drug suspension was then spun in a microfuge at 13,200 rpm for 10 min and the supernatant filtered through Micropure separators (Amicon). After a 1:200 dilution in the same vehicle, the filtrates were evaluated for UV absorption at 245 nm. Drug concentration in the undiluted filtrates, assumed to represent its solubility in the specific vehicle, was calculated as:

$$Cs = A \times df \times 10 / E_1^1$$

where Cs represents drug solubility, A the absorption at 245 nm wavelength, df the factor by which filtrates were diluted (=200), and E_1^1 is the specific absorption coefficient and is =376 (as described above).

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-Pgp-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 (ED_{50}) in the Pgp-positive cells, by interpolation on the dose-response curve. A "MDR1-specific" ED_{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 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).

Doxorubicin accumulation in ascites cells in vivo

NCr *nu/nu* female athymic nude mice were inoculated with either $2x10^6$ MDA435/LCC6 (control) or MDA435/LCC6^{MDR1} *i.p.* After ascites developed, mice were treated with doxorubicin (12 mg/kg) and then sacrificed at 1, 6 and 24 hrs post administration. 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.

Evaluation of *in vivo* MDR1 reversing activity.

The effect on the doxorubicin accumulation in ascites cells was used as the endpoint for the evaluation of PgA4 *in vivo* MDR1-reversing activity. Mice were prepared as described above and, once the ascites had matured, assigned to two major treatment groups (4 mice per group per cell line). One group was treated with PgA4 *s.c.*, as described above for the evaluation of PgA4's *in vivo* toxicity. The other group was treated with an equivalent amount of PgA4 vehicle (20% hydroxypropylcyclodextrin). The mice were treated with doxorubicin 12 mg/Kg *iv* immediately following the 5th of PgA4 inoculations. 24 hours later, they were sacrificed, and the ascites collected and analysed as described above. Two mice per cell line were assigned to treatment with PgA4 only (+ doxorubicin vehicle = saline solution), and two more to no treatment (vehicles only), as controls.

Preparation of immobilized Pgp Artificial Membrane (IAM)

MDA435/LCC6^{MDR1} cells ($\approx 20 \times 10^6$) were harvested in 30 ml of PBS saline and homogenized for 20 sec with a Brinkmann Polytron homogenizer. The homogenates were

centrifuged at 35,000 x g for 10 min and the pellets were suspended in 4 ml solubilization solution (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.5% CHAPS, 2 mM DTT, 5% glycerol) and stirred for 1 hr at 0 °C.

200 mg of dried IAM particles were suspended in 4 ml of the receptor-detergent solution and stirred for 1 hour at 4 °C. The mixture was dialyzed against dialysis buffer (150 mM NaCl, 10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA) for 72 hours at 4 °C. The obtained Pgp-IAM particles were washed with the buffer by centrifugation and packed in a glass column (id 0.5 cm).

Reconstitution and immobilization of PGP on Superdex 200 gel beads

The cultured cells MDA435/LCC6^{MDR1} (20 x 10^6 cells) were harvested in 20 ml of PBS saline and homogenized for 20 sec with a Brinkmann Polytron homogenizer. The homogenates were centrifuged at 35,000 x g for 10 min and the supernatant was discarded. The pellets were suspended in 4 ml solubilization solution [50 mM Tris-HCl, pH 7.5 containing 1.4% octyl β-D-glucopyranoside, 20% glycerol, 1 mM Dithiothreitiol, 1mM benzamidine and 0.4% phospholipid: *E.coli* bulk phospholipid: PC:PS:Cholesterol (60:17.5:10:12.5)] by stirring at 0 °C for 40 min. Non-soluble material was removed by centrifugation. The supernant was applied on to a Sephadex G50 column which equilibrated with elution buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM Benzamidine). The liposome fractions were collected and concentrated to 1 ml. The concentrated liposome solution was mixed with 50 mg dried Superdex 200 and kept in room temperature for 2 hours. The mixture of liposome and Superdex 200 was frozen at -75 °C for 10 min and then thawed at 25 °C for 10 min and the freeze-thaw cycle was repeated. The non-immobilized liposomes were removed by centrifugation and the resulting Pgp-Superdex gel beads were packed in a LC column.

Chromatographic analysis of binding affinity of [^{3}H] VBL at PGP

The PGP-IAM column or PGP-Superdex 200 column was washed with buffer (150 mM NaCl, 10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA). The column was placed in a standard HPLC system. 10-40 ml of [^{3}H] vinblastine with different concentrations in buffer or 1 nM [^{3}H]-vinblastine plus 200 nM ADR in buffer were applied onto the columns at 0.4 ml/min. An on-line flow scintillation detector monitored the elution profile.

Bicinchoninic Acid (BCA) Protein Assay

The Pgp-IAM and Pgp Superdex 200 packing materials were collected and the supernatant were removed. The samples were diluted with 0.1 N of NaOH to 2 ml. A protein standard (0.2-25 μg protein in 50 μl) was prepared with Albumin standard (Pierce). 20 ml of reagent A was mixed with 0.4 ml of reagent B. The standards and samples (50 μl each) were added to triplicate wells in a plate. 200 μl of BCA reagents (A+B) (Pierce) was added in each well. The plate was incubated for 2h at room temperature and read in a spectrophotometer at 570 nm using the Softmax program for the calculation of protein amount.

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

<u>Dehydroprogesterone [30]</u>: *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 a clear solution, and then dried over $MgSO_4$. The solvent was removed under reduced pressure and purified by chromatography; 1.69 g product (35%, R_f = 0.44, 3:2 hexane-ethyl acetate); yellow solid (m.p. = 143-145 °C). ^{1}H NMR: δ 6.12 (s, 1H), 5.69 (s, 1H), 2.84-1.12 (complex, 12H), 2.17 (s, 3H), 2.14 (s, 3H), 1.12 (s, 3H), 1.10 (s, 1H), 1.00 (s, 1H), 0.72 (s, 3H). IR: 3855, 3745, 3678, 2953, 1700, 1663, 1457, 1361, 1223, 875, 754.

7α-[4'-(aminophenyl)thio]-pregna-4-ene-3,20-dione (**PgA 1**) [31]: Dehydroprogesterone (1.65 g, 5.28 mmol), 4-aminothiophenol (1.32 g, 10.56 mmol) and NaOH (pellet, 116 mg, 2.9 mmol) were placed in a Schlenk tube, which was purged with a constant flow of N_2 (g). Deoxgenated 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; 790 mg white solid (61%, R_f = 0.23, 3:2 hexane-ethyl acetate); m.p. = 228-230 °C). ^{1}H NMR: δ 7.26-7.21 (q, J = 8.5 Hz, 2H), 6.64-6.61 (q, J = 8.5 Hz, 2H), 5.73 (s, 1H), 3.77 (s, 2H), 3.24 (s, 1H), 2.14 (s, 3H), 2.63-1.10 (complex, 11H), 1.19 (s, 3H), 0.69 (s, 3H). IR: 3420, 3360, 3250, 2930, 1700. ^{13}C NMR: δ209.3, 199.0, 167.6, 147.1, 136.6, 127.3, 121.2, 115.7, 63.4, 17.7, 13.1.

General Procedure for the Preparation of Progesterone Analogs

A suspension of 7α-[4'-(aminophenyl)thio]-pregna-4-ene-3,20-dione (**PgA 1**) 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.

7α [4'-(N-chloroethylaminoacylaminophenyl)thio]pregna-4-ene-3,20-dione (**PgA 2**): Reaction of **PgA 1** (0.10 g, 0.23 mmol) with 2-chloroethylisocyanate (38 μL, 0.46 mmol) in $CHCl_3$ (3.0 mL) for 12 hrs gave 50 mg product (40%, m.p.=137-141 °C, R_f = 0.47, 2:3 hexane-ethyl acetate). ^{1}H NMR: δ 7.34-7.25 (m, 4H), 5.69 (s, 1H), 5.18 (s, 1H), 3.68-3.62 (m, 4H), 3.38 (s, 1H), 2.64-0.84 (complex, 18H), 2.14 (s, 3H), 1.19 (s, 3H), 0.69 (s, 3H). IR: 3312, 2964, 1700, 1630, 1587, 1517, 1488, 1449, 1394, 1238, 1013, 831, 734. ^{13}C NMR: δ 231.5, 210.3, 196.2, 193.9, 181.9, 156.5, 149.3, 146.4, 141.4, 132.9, 125.1, 119.5, 118.5, 103.2, 94.2, 75.9, 75.8, 71.9, 69.3, 49.0, 35.8, 24.2, 14.4. MS: m/e = 543 (24, M^++1), 507 (10), 313 (27), 230 (23), 185 (50), 149 (69), 125 (57), 119 (23), 107 (38), 105 (48), 91 (50), 81 (50), 57 (73), 55 (100). HRMS: calc'd for $C_{30}H_{39}N_2O_3SCl$ $[M+H]^+$: 543.24481; found: 543.24248.

7α [4'-(N-ethylaminoacylaminophenyl)thio]pregna-4-ene-3,20-dione (**PgA 3**): Reaction of **PgA 1** (0.10 g, 0.23 mmol) with ethylisocyanate (37 μL, 0.46 mmol) in $CHCl_3$ (3.0 mL) for 12 hrs gave 78 mg product (67%, m.p.=130-135 °C, R_f = 0.36, 2:3 hexane-ethyl acetate). ^{1}H NMR: δ 7.36-7.25 (m, 4H), 6.38 (s, 1H), 5.69 (s, 1H), 4.18-4.03 (m, 2H), 3.38-3.26 (m, 2H), 2.67-0.68 (complex, 18H), 2.14 (s, 3H), 2.05 (s, 2H), 1.20 (s, 3H), 0.69 (s, 3H). IR: 3855, 3745, 3678, 3373, 2953, 2359, 1700, 1663, 1539, 1457, 1223. ^{13}C NMR: δ 228.5, 222.5, 193.9, 171.5, 141.5, 135.0, 128.3, 127.0, 123.4, 118.5, 108.7, 96.2, 84.5, 69.3, 67.7, 66.0, 62.6, 52.2, 48.4, 46.3, 43.9, 39.8, 34.1, 22.9, 21.2, 13.4. MS: m/e = 509 (62, M^++1), 438 (8), 313 (32), 196 (47), 125 (100), 117 (57), 97 (52), 95 (85), 79 (68), 71 (59). HRMS: calc'd for $C_{30}H_{40}N_2O_3S$ $[M+H]^+$: 509.28378; found: 509.28372.

7α [4'-(N-α-(+)-methylbenzylaminoacylaminophenyl)thio]pregna-4-ene-3,20-dione

(**PgA 4 (+)**): Reaction of **PgA 1** (0.10 g, 0.23 mmol) with (R)-(+)-α-methylbenzylisocyanate (66 μL, 0.46 mmol) in $CHCl_3$ (3.0 mL) for 12 hrs gave 56 mg product (46%, m.p. = 146-149 °C, R_f = 0.46, 2:3 hexane-ethyl acetate). ^{1}H NMR: δ 7.32-7.25 (m, 9H), 5.79-5.77 (m, 1H), 5.70-5.68 (s, 1H), 4.97-4.92 (m, 1H), 4.13-4.06 (m, 1H), 3.28 (s, 1H), 2.64-1.49 (complex, 18H), 2.14 (s, 3H), 1.45 (d, J = 9.3 Hz, 3H), 1.19 (s, 3H), 0.68 (s, 3H). IR: 3353, 3273, 2949, 2854, 2362, 2340, 1700, 1653, 1595, 1539, 1457, 1460, 1376, 1343, 1159, 1089, 916. ^{13}C NMR: δ 209.4, 199.0, 167.6, 147.0, 136.6, 127.2, 121.2, 115.8, 63.4, 17.7, 13.1 . MS: m/e = 585 (11, M^++1), 135 (12), 125 (20), 105 (100), 103 (22), 91 (29), 77 22), 55 (26). HRMS: calc'd for $C_{36}H_{44}N_2O_3S$ $[M+H]^+$: 585.31506; found: 585.31501.

7α [4'-(N-*p*-toluenesulfonylaminoacylaminophenyl)thio]pregna-4-ene-3,20-dione (**PgA 5**): Reaction of **PgA 1** (0.10 g, 0.23 mmol) with *p*-toluenesulfonylisocyanate (59 μL, 0.46 mmol) in $CHCl_3$ (3.0 mL) for 12 hrs gave 120 mg product (83%, m.p.= 128-132 °C, R_f = 0.29, 2:3 hexane-ethyl acetate). ^{1}H NMR: δ 8.38 (s, 1H), 7.88 (d, J = 8.4 Hz, 2H), 7.80 (d, J = 8.3 Hz, 2H), 7.37-7.25 (m, 4H), 5.70 (s, 1H), 3.36 (s, 1H), 2.67-1.13 (complex, 18H), 2.41 (s, 3H), 2.15 (s, 3H), 1.20 (s, 3H), 0.69 (s, 3H). IR: 3855, 3745, 2359, 1700, 1539, 1457, 1160, 1086, 668. ^{13}C NMR: δ 198.6, 148.6, 141.4, 136.6, 134.6, 129.9, 129.7, 129.6, 127.7, 127.2, 126.4, 120.5, 118.5, 92.4, 76.1, 69.3, 63.3, 52.1, 51.1, 47.0, 46.3, 39.8, 39.4, 38.5, 38.1, 35.4, 34.0, 31.6, 23.7, 22.9, 21.8, 21.1, 17.9, 13.4. MS: m/e = 635 (29, M^++1), 313 (39), 155 (33), 135 (36), 125 (65), 119 (64), 91 (100), 85 (92), 77 (47), 59 (50), 47 (45). HRMS: calc'd for $C_{35}H_{42}N_2O_5S_2$ $[M+H]^+$: 635.26135; found: 635.26130.

7α [4'-(N-α-(-)-methylbenzylaminoacylaminophenyl)thio]pregna-4-ene-3,20-dione (**PgA 4 (-)**): Reaction of **PgA 1** (0.15 g, 0.34 mmol) with (S)-(-)-α-methylbenzylisocyanate (193 μL, 1.37 mmol) in $CHCl_3$ (5.0 mL) for 12 hrs gave 65 mg product (33%, m.p.=146-149 °C, R_f = 0.46, 2:3 hexane-ethyl acetate). ^{1}H NMR: δ 7.32-7.25 (m, 9H), 5.79-5.77 (m, 1H), 5.70-5.68 (s, 1H), 4.97-4.92 (m, 1H), 4.13-4.06 (m, 1H), 3.28 (s, 1H), 2.64-1.49 (complex, 18H), 2.14 (s, 3H), 1.45 (d, J = 9.3 Hz, 3H), 1.19 (s, 3H), 0.68 (s, 3H). IR: 3353, 3273, 2949, 2854, 2362, 2340, 1700, 1653, 1595, 1539, 1457, 1460, 1376, 1343, 1159, 1089, 916. ^{13}C NMR: δ 209.4, 199.0, 167.6, 147.0, 136.6, 127.2, 121.2, 115.8, 63.4, 17.7, 13.1. MS: m/e = 585 (11, M^++1), 135 (12), 125 (20), 105 (100), 103 (22), 91 (29), 77 22), 55 (26). HRMS: calc'd for $C_{36}H_{44}N_2O_3S$ $[M+H]^+$: 585.31506; found: 585.31501.

7α [4'-(N-benzylaminoacylaminophenyl)thio]pregna-4-ene-3,20-dione (**PgA 8**): Reaction of **PgA 1** (0.15 g, 0.34 mmol) with benzylisocyanate (169 μL, 1.37 mmol) in $CHCl_3$ (5.0 mL) for 12 hrs gave 80 mg product (41%, m.p.=185-188 °C, R_f = 0.50, 2:3 hexane-ethyl acetate). ^{1}H NMR: δ 7.40-7.22 (m, 9H), 6.82 (s, 1H), 5.72 (s, 1H), 5.38 (t, J = 6.7 Hz, 1H), 4.41-4.38 (m, 2H), 3.34 (m, 1H), 2.18 (s, 3H), 2.64-1.46 (complex, 18H), 1.24 (s, 3H), 0.84 (s, 3H). IR: 3750, 3725, 3429, 2943, 2870, 1710, 1660, 1578, 1450, 1210, 1010. MS: m/e = 571 (34, M^++1), 570 (28), 313 (26), 258 (24), 241 (41), 154 (61), 147 (22), 136 (66), 129 (14), 125 (100), 124 (70), 109 (31). HRMS: calc'd for $C_{35}H_{42}N_2O_3S$ $[M+H]^+$: 571.29569; found: 571.29944.

7α [4'-(N-phenylaminoacylaminophenyl)thio]pregna-4-ene-3,20-dione (**PgA13**): Reaction of **PgA 1** (50 mg, 0.11 mmol) with phenylisocyanate (43 μL, 0.4 mmol) in $CHCl_3$ (3.0 mL) for 12 hrs gave 52 mg product (83%, m.p.=227-230 °C, R_f = 0.54, 2:3 hexane-ethyl acetate).

^{1}H NMR: δ 7.67-7.18 (m, 9H), 7.14 (s, 1H), 5.72 (s, 2H), 3.38 (m, 1H), 2.65-1.24 (complex, 18H), 2.18 (s, 3H), 1.30 (s, 3H), 0.68 (s, 3H). IR: 3500, 3319, 2945, 2851, 1700, 1655, 1585, 1540, 1419, 1380, 1270, 1190. MS: m/e = 557 (3, M^{+}+1), 425 (3), 214 (18), 213 (100), 197 (6), 115 (9), 101 (15). HRMS: calc'd for $C_{34}H_{40}N_2O_3S$ $[M+H]^+$: 557.28381; found: 557.28383.

7α[4'-(N-β-phenylethylaminoacylaminophenyl)thio]pregna-4-ene-3,20-dione (**PgA12**): Reaction of **PgA 1** (90 mg, 0.21 mmol) with β-phenylethylisocyanate (116 μL, 0.84 mmol) in $CHCl_3$ (5.0 mL) for 12 hrs gave 26 mg product (21%, m.p.=126-130 °C, R_f = 0.79, 2:3 hexane-ethyl acetate). ^{1}H NMR: δ 7.36-7.20 (m, 9H), 6.32 (s, 1H), 5.67 (s, 1H), 4.71 (m, 1H), 3.48 (m, 2H), 3.35 (m, 1H), 2.81 (t, J = 6.6 Hz, 2H), 2.68-1.24 (complex, 18H), 2.14 (s, 3H), 1.20 (s, 3H), 0.69 (s, 3H). IR: 3630, 3420, 3024, 2945, 1700, 1640, 1580, 1468, 1459, 1230, 1010. MS: m/e = 585 (9, M^{+}+1), 313 (13), 159 (7), 149 (16), 135 (10), 125 (28), 119 (23), 105 (100), 103 (23). HRMS: calc'd for $C_{36}H_{44}N_2O_3S$ $[M+H]^+$: 585.31506; found: 585.31644.

7α [4'-(N-trifluoro *p*-tolyaminoacylaminophenyl)thio]pregna-4-ene-3,20-dione (**PgA28**): Reaction of **PgA 1** (90 mg, 0.21 mmol) with α,α,α-trifluoro *p*-tolylisocyanate (120 μL, 0.84 mmol) in $CHCl_3$ (5.0 mL) for 12 hrs gave 28 mg product (21%, m.p.=248-252 °C, R_f = 0.67, 2:3 hexane-ethyl acetate). ^{1}H NMR: δ 7.98-7.38 (m, 9H), 5.78 (s, 2H), 3.40 (m, 1H), 2.62-1.26 (complex, 18H), 2.16 (s, 3H), 1.34 (s, 3H), 0.74 (s, 3H). IR: 3820, 3720, 3600, 3024, 2968, 2873, 1705, 1657, 1520, 1437, 1417, 1074, 908, 820. MS: m/e = 625 (50, M^{+}+1), 531 (28), 447 (16), 313 (23), 167 (10), 149 (100), 141 (38), 125 (14), 105 (37). HRMS: calc'd for $C_{35}H_{39}N_2O_3SF_3$ $[M+H]^+$: 625.27118; found: 625.27217.

7α [4'-(N-*p*-tolyaminoacylaminophenyl)thio]pregna-4-ene-3,20-dione (**PgA20**): Reaction of **PgA 1** (90 mg, 0.21 mmol) with 4-methylphenylisocyanate (106 μL, 0.84 mmol) in $CHCl_3$ (5.0 mL) for 12 hrs gave 21 mg product (18%, m.p.=244-250 °C, R_f = 0.45, 2:3 hexane-ethyl acetate). ^{1}H NMR: δ 7.38-7.08 (m, 8H), 7.18 (s, 1H), 5.72 (s, 2H), 3.36 (m, 1H), 2.68-1.24 (complex, 18H), 2.38 (s, 3H), 2.16 (s, 3H), 1.21 (s, 3H), 0.66 (s, 3H). IR: 3540, 3480, 3310, 2965, 2870, 1700, 1655, 1580, 1530, 1420, 1360, 1210. MS: m/e = 571 (45, M^{+}+1), 447 (11), 313 (19), 281 (11), 258 (15), 207 (17), 167 (13), 152 (16), 149 (100), 147 (31), 141 (31), 135 (25), 133 (23), 125 (26), 119 (40), 107 (24). HRMS: calc'd for $C_{35}H_{42}N_2O_3S$ $[M+H]^+$: 571.29944; found: 571.29676.

7α['-(N-*p*-methylbenzylaminoacylaminophenyl)thio]pregna-4-ene-3,20-dione (**PgA11**): Reaction of **PgA 1** (150 mg, 0.34 mmol) with 4-methylbenzylisocyanate (202 mg, 1.37 mmol) in $CHCl_3$ (5.0 mL) for 12 hrs gave 69 mg product (35%, m.p.=145-146 °C, R_f = 0.65, 2:3 hexane-ethyl acetate). ^{1}H NMR: δ 7.58-7.04 (m, 8H), 7.08 (s, 1H), 5.88 (m, 1H), 5.62 (s, 1H), 4.36 (m, 2H), 3.32 (m, 1H), 2.62-1.22 (complex, 18H), 2.36 (s, 3H), 2.12 (s, 3H), 1.21 (s, 3H), 0.66 (s, 3H). IR: 3650, 3600, 3310, 3020, 2985, 2885, 1705, 1650, 1570, 1430, 1201, 1120. MS: m/e = 585 (8, M^{+}+1), 313 (12), 269 (9), 135 (5), 125 (26), 120 (14), 105 (100), 103 (9). HRMS: calc'd for $C_{36}H_{44}N_2O_3S$ $[M+H]^+$: 585.31506; found: 585.31595.

X-ray Crystallographic Analysis

Single crystals of PGA13 were obtained from diethyl ether by slow evaporation. The X-ray crystallographic analysis was carried out at the Department of Chemistry in Georgetown University by Dr. Michael Dickman using a Siemens SMART CCD single-crystal

diffractometer. A sphere of data (97% coverage to q=27^0) was collected using omega scans. Routine Lorentz and polarization corrections were applied. Programs used were SMART, SAINT, and SHELXTL [32-34]. A representation of PgA13's crystal structure is provided in Fig 1. A summary of parameters for data collection and refinement are as follows:

Empirical formula	$C_{34}H_{40}N_2O_3S$	
Formula weight	556.74	
Temperature	179(2) K	
Wavelength	0.71073 Å	
Crystal system	orthorhombic	
Space group	$P2_12_12_1$	
Unit cell dimensions	a = 16.3557(2) Å	$\alpha = 90°$
	b = 19.0634(2) Å	$\beta = 90°$
	c = 19.4147(2) Å	$\gamma = 90°$
Volume, Z	6053.41(12) Å^3, 8	
Density (calculated)	1.222 Mg/m^3	
Absorption coefficient	0.143 mm^{-1}	
F(000)	2384	
Crystal size	0.50 x 0.18 x 0.18 mm	
θ Range for data collection	1.50° to 28.28°	
Limiting indices	$-21 \leq h \leq 21$, $-24 \leq k \leq 25$, $-25 \leq 1 \leq 25$	
Reflections collected	66127	
Independent reflections	14393 (R_{int} = 0.0728)	
Refinement method	full-matrix least-squares on F^2	
Data/restraints/parameters	14393/0/727	
Goodness-of-fit on F^2	0.991	
Final R indices [$I > 2\sigma(I)$]	R1 = 0.0526, wR2 = 0.1046	
R indices (all data)	R1 = 0.1099, wR2 = 0.1285	
Absolute structure parameter	-0.04(6)	
Largest diff. Peak and hole	0.185 and 0.267 e Å^{-3}	

RESULTS AND DISCUSSION

OBJECTIVE 1:

IN VIVO EVALUATION OF PGA4'S TOXICITY AND MDR1 REVERSING ACTIVITY

Doxorubicin accumulation assay for the preliminary evaluation of *in vivo* MDR1-reversing activity

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). MDR1-reversing agents are known to potentially affect the MDR1 anticancer substrates pharmacokinetics (and toxicity) through interaction with Pgp expressed in normal tissues [24, 35]. Consequently, the tests will be carried out comparing equitoxic doses of the anticancer drug in the absence and presence of the test agent and will require a preliminary evaluation of the test compound effect on the anticancer drug MTD.

In order to optimally exploit our resources and reduce the use of animals, we decided to reserve tumor growth delay and % ILS assays to test one (or a very few) selected drugs for which some preliminary evidence of *in vivo* activity is available. We believe that a good preliminary endpoint of *in vivo* MDR1-reversing activity is represented by doxorubicin accumulation in cancer cells grown and treated *in vivo*. The mechanism of action of Pgp, the delayed time of doxorubicin lethality and the ease with which doxorubicin content can be analyzed exploiting its fluorescence, all support our choice. In this assay, the parallel use of the parental MDR1-negative cells as control can help discriminate modifications in doxorubicin accumulation due to pharmacokinetic modulation rather than to interaction with the Pgp expressed on cancer cells.

In vitro evaluations have confirmed that MDR1 expression confer MDA435/LCC6^{MDR1} cells the ability to maintain lower intracellular levels of the anticancer substrates doxorubicin as compared to the parental cell line MDA435/LCC6 (Fig. 2). We have observed a 6.5-fold difference (mean of 5 separate determinations, +/- 0.7 SE) in intracellular accumulation of the drug under *in vitro* conditions after treating cells with doxorubicin 4 μM for 3 hours. This difference is completely reversed by treatment with MDR1-reversing agents (see below, and Fig. 6).

We assessed doxorubicin accumulation in ascites cells in relation to time. NCr *nu/nu* female athymic nude mice were inoculated *i.p.* with either LCC6/MDR1 or LCC6 cells and, after the appearance of ascites, treated with doxorubicin 12 mg/Kg *i.v.* Ascites were collected at 1, 6 and 24 hours after doxorubicin treatment and the cell sediment analyzed for doxorubicin content. The results confirm that LCC6 and LCC6/MDR1 cells grown and treated *in vivo* as ascites accumulate doxorubicin differentially. The difference is not evident at 1 hour, but becomes apparent at 6 hours (2.4-fold), and is maximal at 24 hours (10.8-fold) (Fig. 3). We are now in the process of reevaluating the time-dependence of doxorubicin accumulation in solid tumors originated from the same cell lines, using an extended number of time points. We also have decided to adopt the doxorubicin accumulation endpoint in ascites cells for the preliminary evaluation of PgA4's *in vivo* MDR1-reversing efficacy.

Formulation studies

Like most known MDR1-reversing agents, PgA4 appears to have a limited solubility in water (Table 1). To optimize PgA4 formulation and maximize the dose that can be delivered *in vivo*, we evaluated PgA4 solubility in different solvents. Ethanol, DMSO, propylene glycol and glycerin are all solvents used in classical formulations. However, because of their local or systemic toxicity they must be used in combination with other solvents, *e.g.*, the use of ethanol at concentrations higher than 10% is contraindicated, while DMSO's LD_{50} (acute

toxicity following *iv* administration) in mice is about 6 g/Kg [36]. Cyclodextrins appear to effectively increase the solubility of steroids and other lipophilic drugs by the formation of inclusion complexes. They are relatively non-toxic: 2-hydroxypropyl-β-cyclodextrin 10 g/Kg *i.p.* caused no death among four mice [37].

In preliminary evaluations (carried out in methanol), we characterized PgA4 UV profile. PgA4's Specific Absorption Coefficient ($E_1^{\ 1}$) at 245 nm wavelength is 376.1+/- 4.3 SE (average of 3 determinations).

We defined PgA4 solubility in water, ethanol, DMSO, propylene glycol, glycerol and 20% 2-hydroxypropyl-β-cyclodextrin (HPCD). Our results (reported in Table 1) suggest maximum solubility in ethanol and DMSO, with good solubility in propylene glycol. Solubility in HPCD, though more than 10 times lower than in ethanol, appeared the most promising, as HPCD represents a complete formulation. In a direct comparison, 20% HPCD appeared superior to a classical formulation (10% ethanol; 40% propylene glycol), the solubility of PgA4 being respectively 2.4 and 0.4 mg/ml in the two vehicles.

To exclude the possibility that the interaction with the cyclodextrin may affect PgA's action on Pgp, we compared the ability of PgA4 prepared from HPCD and ethanol stock solutions to increase doxorubicin in LCC6/MDR1 cells *in vitro*. By demonstrating that the biological activity of drug preparations from the two stocks is equivalent (Fig. 4), the results confirm that HPCD does not alter PgA4's interaction with Pgp.

Based on the above results, we selected 20% HPCD as the PgA4 formulation vehicle for all subsequent *in vivo* studies.

Evaluation of PgA4's *in vivo* toxicity and MDR1-reversing activity

PgA4 toxicity. To maximize our ability to detect any MDR1-reversing effect, and as PgA4 pharmacokinetics are presently unknown, we decided to use a treatment regimen that would "load" the test animals with PgA4 prior to and during treatment with the anticancer drug. The maximum deliverable dose of PgA4 was dictated by PgA4 solubility in HPCD as well as constraints in the amount of solution that can be delivered *s.c.* We determined to treat the animals with PgA4 15 mg/Kg (10 μl/g body weight of a solution of PgA4 1.5 mg/ml in HPCD), twice a day for 3 days.

Prior to testing its effect on doxorubicin accumulation, we tested the toxicity of our PgA4 treatment regimen on a limited number of mice of the same strain (2 mice per treatment arm), using treatment with vehicle as control. This treatment showed neither immediate effect in terms of mortality, or altered behavior, nor sign of local toxicity. Moreover, a longer term evaluation showed no significant effect of PgA4 treatment on the animal body weight gain up to 55 days following the completion of treatment (Fig.5).

PgA4 in vivo MDR1 reversing activity. Following inoculation *i.p.* of LCC6 or LCC6/MDR1

cells and the appearance of ascites, mice were assigned to two major treatment arms (5 mice per arm, planned), to be treated either with doxorubicin plus PgA4 or with doxorubicin with PgA4 vehicle only. PgA4 (or vehicle) treatments were carried out as described above and doxorubicin was inoculated *i.v.*, at a dose of 12 mg/Kg concurrent with the 5th inoculation of PgA4. The animals were sacrificed and the ascites collected 24 hours later. The evaluation of MDR1-reversing activity failed to give meaningful results, as we incurred into unexpected problems with our ascites model. Contrary to our previous experience, LCC6 and LCC6/MDR1 cells produced ascites over too wide a time span and, in a couple of cases, failed to produce ascites altogether. Moreover, we observed large differences in doxorubicin accumulation and no important overall difference between MDR1-positive and -negative ascites. We think that these results might be due to changes in the characteristics of our cell stock.

Future directions

We are evaluating the doxorubicin accumulation LCC/MDR1 *vs* LCC6 solid tumors which were collected at different time points (1, 6, 24, 48 and 72 hours) following inoculation with doxorubicin *iv*. To allow for direct copmparison with other published studies, we also plan to use the ascites-producing murine leukemia cell line P388 and its MDR1-positive derivative, P388/ADR [38, 39]. Several Pgp reversing compounds have been published in this model.

OBJECTIVES 2 AND 3: BRIDGE AND F-RING OPTIMIZATION AND *IN VITRO* POTENCY

We have begun to evaluate the structure-activity data generated with our expanding series of compounds. We now present our initial observations and will expand the analyses, incorporating additional data, in year 3. It should be noted that we have not had sufficient time to fully explore our existing data, and that many of the comments provided below are somewhat speculative at present.

To test the role of bridge length and F-ring in the PgA's MDR1-reversing activity, we have synthesized seven new progesterone analogs by reacting PgA1 with different isocyanates. An *in vitro* doxorubicin accumulation endpoint was used to provide an initial measure and comparison of the MDR1-reversing activity and potency of first and second generation PgA. The results of these evaluations are summarized in Table 2 in terms of potency relative to PgA4, and illustrated in Fig. 6. The mean ED_{50} values of PgA4 for reversal of doxorubicin accumulation are 641.5 +/- 93.2 SE and 1000.7 +/- 74.5 SE, respectively before and after correction for non-MDR1-specific effect. Table 2 also provides a measure of each drug's hydrophobicity (as evaluated in terms of Retention Factor, by TLC) to support some preliminary structure-activity considerations.

Role of F-ring and bridge length.

The presence of an aromatic F ring confers more than a 3-fold increase in MDR1 cell-specific doxorubicin accumulation effect, 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 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. The increased potency conferred by the 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.

Role of the E-ring/urea group.
The C7 chain of the simplest of PgA, PgA3 (where the susbstituent distal to the urea group is an ethyl group) confers a 29-fold potentiation of the MDR1-reversing activity of Progesterone. By comparison, further modification of the side chain distal to the urea group has so far conferred a further increase of such activity up to 3.4 fold, as compared to PgA3. This evidence suggests a comparatively major role for the E ring and/or the attached urea group in potentiating MDR1-inhibition. This increase in activity does not appear to be due to changes in overall hydrophobicity, a factor which correlates with the activity of smaller steroids [7], as this this is lower in all PgA than in Progesterone (Table 2).

Role of hydrophobicity.
Contrary to what has been suggested for simpler steroids [7], there is no significant correlation between the MDR1-reversing potency and the hydrophobicity across different PgA (Table 2).

Future directions

(A) Generation of new PgA compounds for structure-activity relationship studies and optimization of MDR1 reversing activity.
(1) Modification of the portion of the C7 side chain distal to the urea group (Scheme 2). Presently, more analogs are being obtained by reaction of PgA1 with different isocyanates. We intend to further define the following points: (1a) the role of carbon chain length in the absence of an aromatic F ring (analogs of PgA3 by addition or subtraction of –CH2-groups: compounds *a-d* in Scheme 2); (1b) the role of a partial positive or negative charge on the F ring (we will compare the potency of electron-withdrawing and electron-donating substituents in *para* position: compounds *e-g* in Scheme 2), and (1c) the effect of the presence of an additional aromatic ring (ring G) attached to the F ring (compounds *h* in Scheme 2).
(2) Synthesis of compounds to test the role of the urea group and of the E ring in the C7 side chain (Scheme 3). We will generate analogs of PgA3 deprived of either the urea and/or the E ring. The role of these two groups will be tested by comparing the activity of PgA3 with respectively compounds *i* and *l, m* in Scheme 3. Compounds *n,o* are designed to test the combined effect of E ring and urea group.

(3) Synthesis of compounds to test the effect of the relative position of E ring and urea groups in the C7 side chain (Scheme 4). (3a) The *para* position of the urea substituent on the E ring may not be optimal for MDR1 reversing activity. To test this hypothesis, we will generate PgA3 analogs with urea in the *ortho* and *meta* positions (compounds *p, r* and *q, s* in Scheme 4). (3b) We will also test the effect of increasing the distance between E ring and urea group. The activity of compounds *t* and *u* in Scheme 4 will be compared with that of the compounds *c* and *d* (Scheme 2), which have the same overall C7 group length.
(4) Substitution of sulfur with a carbon bridge in the C7 side chain (Scheme 5). In last year's report, we speculated about the possible role of the sulfur bridge on the C7 side chain in making PgA particularly vulnerable to hepatic metabolism. Preliminary attempts to create PgA analogs with a carbon in place of the sulfur bridge have been unsuccessful. We will pursue this objective with the help of our newly hired postdoctoral fellow (Dr. Ji-Hyun Kim; see attached Biosketch). Following synthesis of the C-bridge analog of PgA4, we will compare the *in vitro* activity of the two compounds before and after exposure to liver microsomal preparations. If this preliminary test confirms a higher metabolic stability, we will then proceed to the evaluation of the MDR1-reversing efficacy *in vivo*.
(5) Role of polar substitutions in C3 and C17. The results of a study of steroid structure-activity correlations [7] suggest that the presence of a hydroxyl group in C21, may increase the MDR1-reversing potency/hydrophobicity ratio. Hydroxylation may also help to make the drug more soluble in aqueous formulations and so increase our ability to deliver it *in vivo*. We also may decide to synthesize analogs with a sulfate substituent in the C3 or C20 position.

(B) Evaluation of MDR1-reversing spectrum.
Structure–activity relationships of PgA's interaction with MDR1 anticancer drug substrates may differ with the anticancer drug, possibly due to the existence of partially or not overlapping binding sites. Consequently, all drugs will be tested for their effect on vinblastine and taxol, beside doxorubicin, accumulation. However, only for selected compounds will activity be confirmed using more involving chemosensitization experiments, *i.e.,* using a cytotoxicity endpoint and isobologram analysis (as already described for PgA4).

(C) Evaluation of the correlation of PgA compounds' hydrophobicity and lipophilicity with their MDR1-reversing activity/potency.
All analogs will be evaluated for general hydrophobicity (by TLC, as described above) and lipophilicity (in terms of water-octanol partition coefficient). Results will be used to discriminate the role of these properties from that of 3-dimensional (shape and charge distribution) factors. The ability of a compound to reach its binding site on Pgp, inside the cell membrane, may be more crucially affected by its lipophilicity than by its hydrophobicity [40]. We plan to evaluate the correlation between the MDR1-reversing activity of our compounds and their water-octanol partition coefficient.

OBJECTIVE 5: MECHANISM OF MDR1-REVERSAL BY PGA COMPOUNDS

We have initially hypothesized that the PgA compounds reverse the MDR1 phenotype by

competing with anticancer substrates for the Pgp binding site. Testing of this mechanistic hypothesis has been set for the 3rd to 4th year of this grant (Months 30-48).

Evidence from the literature supports such hypothesis. Steroidal compounds have been shown both to reverse the MDR1 phenotype and to be actively transported by Pgp, the former activity appearing directly and the latter inversely proportional to hydrophobicity [7, 25, 41-43]. Moreover, photoaffinity labeling studies demonstrate direct binding of steroids to Pgp [42, 44].

It is presently unclear whether Pgp binds its substrates at one or more binding sites. Studies using classical membrane binding assays and the analysis of dissociation rates have shown that some substrates, though interacting "competitively", might actually bind to distinct, though allosterically connected, sites [10, 11]. It may be speculated that the PgA binding site(s) may, either partially or totally, overlap with those of some anticancer substrates and be only allosterically connected to others. It is also possible that MDR1 susbtrates bind to shallow sites on an extended binding surface and that partial overlapping may be affected by Pgp conformation as determined by the specific membrane environment.

To address the nature of the PgA compounds' interaction with Pgp anticancer susbtrates, we have now decided to use a chromatographic approach, as opposed to classical binding assays using membrane suspensions. In the chromatographic approach, the binding sites are immobilized in a column's stationary phase. This offers the advantage of chromatography precision and reproducibility and can be easily used for the rapid on-line screening of combinatorial drug pools and the evaluation of quantitative structure-activity relationships [45, 46], making it particularly suited for the drug discovery process. Dr. Irving Wainer, who has recently joined our effort on this Project (see attached Biosketch), is well known for his experience with this approach, which he has used to study, among other subjects, chymotrypsin enzymatic and nicotinic receptor binding activities [47, 48].

In collaboration with Dr. Wainer, we have now obtained for the first time a Pgp column for the study of substrate interactions and have started its characterization

Initial characterization of the Pgp column

An evaluation of protein content showed that, for one milliliter of bed volume, about 170 mg proteins were immobilized on the IAM column and about 10 mg proteins were immobilized on Superdex 200 column. The chromatographic results obtained with the Pgp-IAM column or Pgp-Superdex 200 column indicated that the binding activity of Pgp was retained after immobilization. For example, [^{3}H]-vinblastine was retarded on a Pgp-IAM column (0.5 x 0.8 cm) and the retention volume was 13.3 ml at the concentration of 1 nM (profile A in Fig 7) at flow rate: 0.4 ml/min. When a displacer ligand, doxorubicin (200 nM), was included in the mobile phase, the retention volume of 1 nM [^{3}H]-VBL was decreased from 13.3 ml to 6.5 ml (profile B in Fig.7). This indicated that the retardation was partially due to the specific binding to saturable binding sites of PGP.

Frontal chromatography can be used to calculate dissociation constants, K_d, for the marker and displacer ligands. The experimental approach involves the variation of the concentrations of Tracer (T= a fluorescent or radio-labeled ligand)) and the competitive ligands in the mobile phase. From the resulting data, the association constants of T, K_T, and the test drug, K_{drug}, as well as the number of the active and available binding sites of immobilized receptors, P, can be calculated using the following equations, Eqn 1 and Eqn 2.

$$(V_{max} - V)^{-1} = (1+[T]K_T)(V_{min}[P]K_T)^{-1} + (1+[T]K_T)^2 (V_{min}[P]K_T K_{drug})^{-1}[drug]^{-1}$$
(Eqn 1)

$$(V - V_{min})^{-1} = (V_{min}[P]K_T)^{-1} + (V_{min}[P])^{-1}[T]$$
(Eqn 2)

In the above equations, V is the retention volume of T; V_{max}, the retention volume of T at low concentration (60 pM) and in the absence of drugs; V_{min}, the retention volume of T when the specific interaction is completely suppressed. The value of V_{min} is determined by running T in a series of concentration of drugs and plotting $1/(V_{max} - V)$ *versus* 1/[drug] extrapolating to infinite [drug]. From the above plot and a plot of $1/(V - V_{min})$ *vs.* [T], dissociation constant values, K_d, for T and the drugs are obtained.

The retention volumes of $[^3H]$-vinblastine at the different concentration in frontal chromatography and Eqn 1 and 2 were used to calculate the K_d value. **The obtained K_d value for $[^3H]$-vinblastine determined in this technique is 19 ± 20 nM, that is consistent with the reported value, 36 ± 55 nM** (mean ± SD) [49]. These preliminary results indicate that Pgp-based chromatographic stationary phase can be used for the investigation of Pgp-substrate interactions.

Future directions

We will proceed with our column characterization. We will compare binding in columns where Pgp is reconstituted in IAM *vs* proteoliposomes. If necessary, we will use purified Pgp. We will optimize ionic strength and pH of the mobile phase and assess stability and reproducibility of Pgp columns and the effect of the absence/presence of ATP on binding characteristics.

Using the optimized column, we will evaluate the interaction between the PgA compounds and MDR1 main anticancer substrates (doxorubicin, taxol, vinblastine, etoposide), to test the competitive nature of interaction and whether the respective binding sites are overlapping.

CONCLUSIONS

We have defined PgA4 formulation for *in vivo* treatment and established an approach for the preliminary evaluation of our test compounds' *in vivo* MDR1 reversing efficacy. Studies of the *in vivo* activity are still in progress, and we expect to make major advances in this area in year 3.

We have synthesized and tested several new analogs of progesterone by reaction of Pg1 with different isocyanates. The most potent of our analogs (PgA13) is now about 95-fold more potent than the parental compound progesterone in reversing the MDR1 phenotype (using a doxorubicin accumulation endpoint). The aromatic F ring appears to effectively increase MDR1-reversing activity (in terms of doxorubicin accumulation), but only in the analogs where the aromatic F ring is directly connected to the urea group (phenyl substitution on the urea group). This effect is partially or completely lost when this distance is increased (e.g., benzyl and phenethyl substitution on the urea group). The effect of the F ring is also lost when it is made relatively more positive by substitution with an electron withdrawing group. Most of the gain in MDR1-reversing potency appears still to be associated with the proximal portion of PgAs' C7 side chain, *i.e.*, its E ring and/or urea groups. The new series of analogs that we will attempt to synthesize during the next year, will further test structure-activity relationships and optimize MDR1 reversing activity. The new analogs will be obtained by a step-by-step modification of the distal and proximal portions of the C7 side chain and by studying the effect of substitutions in the C17 and C3 positions of the steroid nucleus.

Different MDR1 substrates may bind to different sites or localities in Pgp. We will define whether PgA share binding sites with different anticancer substrates and evaluate the structure-activity relationship of PgA's interaction with such substrates (specifically, doxorubicin, etoposide, vinblastine and taxol). To further define the determinants of MDR1 activity, all analogs will be characterized for hydrophobicity and lipophilicity.

Our most promising leads will be preliminarily tested for *in vivo* activity using our *in vivo* doxorubicin accumulation assay. We have defined a formulation for the *in vivo* administration of PgA4 using a cyclodextrin solution as vehicle. Administration of PgA4 twice a day for three days at nearly the maximally achievable dose has shown no evidence of significant acute or subacute toxicity in preliminary evaluations. One or a very restricted number of agents active in the preliminary studies will be tested for a definitive evaluation of their *in vivo* efficacy using a %ILS or tumor growth delay assay, using equitoxic concentrations of the reference anticancer drug.

REFERENCES

1. B. Trock, F. Leonessa, R. Clarke, *J Natl Cancer Inst* 89: 917-931, 1997

2. Y. Raviv, H.P. Pollard, E.P. Bruggemann, I. Pastan, M.M. Gottesman, *J Biol Chem* 265: 3975-3980, 1990

3. M.M. Gottesman, *Cancer Res* 53: 747-754, 1993

4. J.M. Ford and W.N. Hait, *Pharmacol Rev* 42: 155-199, 1990

5. W.T. Beck and X.-D. Qian X-D, *Biochem Pharmacol* 43: 89-93, 1992.

6. U.S. Rao and G.A. Scarborough, *Mol Pharmacol* 45: 773-776, 1994

7. K.M. Barnes, B. Dickstein, G.B. Cutler.Jr., T. Fojo and S.E. Bates, *Biochemistry* 35: 4820-4827, 1996.

8. T. Saeki, K. Ueda, Y. Tanigawara, R. Hori and T. Komano, *J Biol. Chem* 268: 6077-6080, 1993.

9. A. Sakata, I. Tamai, K. Kawazu, Y.Deguchi, T. Ohnishi , A. Saheki and A. Tsuji, *Biochem Pharmacol* 48: 1989-1992, 1994.

10. D.R. Ferry, M.A. Russell, M.H. Cullen, *Biochem Biophys Res Comm* 188:440-445, 1992.

11. D.R. Ferry, P.J. Malkhandi, M.A. Russell, D.J. Kerr, *Biochem Pharmacol* 49:1851-1861, 1995.

12. J. Malkhandi, D.R. Ferry, R. Boer, V. Gekeler, W. Ise, D.J. Kerr, *Eur J Pharmacol* 288:105-114, 1994.

13. D.R. Ferry, H. Traunecker, and D.J. Kerr, *Eur J Cancer* 32A: 1070-1081, 1996

14. T.P. Miller, T.M. Grogan, W.S. Dalton, C.M. Spier, R.J. Scheper, S.E. Salmon. *J Clin Oncol* 9: 176-24, 1991

15. R.D. Christen, E.F. McClay, S.C. Plaxe, *et al*: PhaseI/pharmacokinetic study of high-dose progesterone and doxorubicin. *J Clin Oncol* 11: 2417-2426, 1993

16. D.L. Trump, D.C. Smith, P.G Ellis *et al*. *J Natl Cancer Inst* 84: 1811-1816, 1992

17. P. Sonneveld, B.G.M. Durie, G.M. Lokhorst, *et al,* Lancet 340: 255-258, 1992

18. C.J. Punt, E.E. Voest, E. Tueni , *et al, Br J Cancer* 76: 1376-1381, 1996

19. B. Tranchant, G. Catimel, C. Lucas, *et al, Cancer Chemother Pharmacol* 41: 281-291, 1998

20. V. Nuessler, M.E. Scheulen, R. Oberneder *et al, Eur J Med Res* 2: 55-61, 1997

21. E. Warner, D. Hedley, I. Andrulis, *et al, Clin Cancer Res* 4: 1451-1457, 1998

22. P. Sonneveld, J.P. Marie, C. Huisman, *et al, Leukemia* 10: 1741-1750, 1996

23. S.M. Kornblau, E. Estey, T. Madden, *et al, J Clin Oncol* 15: 1796-1802, 1997

24. D.J. Boote, I.F. Dennis, P.R Twentyman *et al, J Clin Oncol* 14: 610-618, 1996

25. M. Naito, K. Yusa, T. Tsuruo, *Biochem Biophys Res Comm* 158: 1066-1071, 1989

26. B. Beyer, L. Terenius, R.E. Counsell, *Steroids* 35: 481-488, 1980

27. F. Leonessa, D. Green, T. Licht, A. Wright, K. Wingate-Legette, J. Lippman, M.M. Gottesman, R. Clarke, *Br J Cancer* 73: 154-161, 1996

28. D.F. Shriver and M.A. Drezdzon, in *The Manipulation of Air-Sensitive Compounds*, 2nd ed.; Wiley: New York, 1986.

29. W.C. Still, M. Kahn, A. Mitra, *J Org Chem* 43: 2923-2925, 1978

30. R.K. Haynex, W.R. Jackson, S. Stragalinou, *Aust J Chem* : 1537-1544, 1980

31. R.W. Brueggemeier, E.E. Floyd, R.E. Counsell, *J Med Chem* 21: 1008-1011, 1978

32. G.M. Sheldrick, in *SHELXTL Structure Determination Software Program*; Siemens Analytical X-ray Instruments, Inc.: Madison, WI, 1990.

33. G.M. Sheldrick, *Acta Crystallogr* A46: 467-473, 1990

34. *SMART* and *SAINT* User's Manual, Version 4.050;Siemens Analytical X-ray Instruments, Inc.: Madison, WI, 1996.

35. S.E.Bates, W.H. Wilson, A.T. Fojo, *et al, Stem Cells* 14: 56-63, 1996

36. J.E. Willson, D.E. Brown, and E.K. Timmens, *Toxicol Appl Pharmacol* 7: 104-112, 1965

37. J. Pitha and J. Pitha, *J Pharm Sci* 74: 987-990, 1985

38. R.K. Johnson, M.P. Chitnis, W.M. Embrey, and E.B. Gregory, *Cancer Treat Rep* 62:

1535-1547, 1982

39. D. Kessel, K. Woodburn, Br J Cancer 71: 306-310, 1995

40. G. Ecker, P. Chiba, M. Hitzler, *et al, J Med Chem* 39: 4767-4774, 1996

41. C.-P.H. Yang, S.G. DePinho, L.M. Greenberger, R.J. Arceci, and S.B. Horwitz, *J Biol Chem* 264: 782-788, 1989

42. D.C. Wolf, and S.B. Horwitz, *Int J Cancer* 52: 141-146, 1992

43. C.K. van Kalken, H.J. Broxterman, H.M. Pinedo, N. Feller, H. Dekker, J. Lankelma, and G. Giaccone, . *Br J Cancer* 67: 284-289, 1993

44. X.-D. Qian, and W.T. Beck, *J Biol Chem* 265: 18753-18756, 1990

45. I.W. Wainer, M. Crabos, J.F. Cloix, *J Chromatogr* 338: 417-421, 1985

46. R. Kaliszan, T.A.G. Noctor, and I.W. Wainer, *Molec Pharmacol* 42: 512-527, 1992

47. T. Alebic-Kolbah, and I.W. Wainer, *J Chromatogr A* 653: 122-129, 1993

48. Y. Zhang, Y. Xiao, K.J. Keller, I.W. Wainer, *J Chromatogr B*, in press.

49. R. Callaghan, G. Berridge, D.R. Ferry, and C.F. Higgins, *Biochem Biophys Acta* 1328-109-124, 1997

Scheme 1. Synthesis of PgA compounds

1 → 2: DDQ, PhH, reflux

2 → 3: 4-aminothiophenol (SH / NH_2), Dioxane

3 → 4: RNCO, $CHCl_3$

3: S–C_6H_4–NH_2

4: S–C_6H_4–NHCONHR

Scheme 2. Further modifications to the distal portion of the C7 side chain

PgA1

RNCO, $CHCl_3$

NH_2

NHCONHR

R:

a $-CH_3$

b

c

d

e

f

g NO_2

h

Scheme 3. Synthesis of compounds to test the role of urea group and E ring

Dehydroprogesterone

$HS(CH_2)_nNH_2$

k

$S—(CH_2)_NNH_2$

CH_3CH_2NCO

$S—(CH_2)_NNHCONHCH_2CH_3$

n = 2 (*l*), 3 (*m*)

$HS—C_6H_4—(CH_2)_3CH_3$

$S—C_6H_4—(CH_2)_3CH_3$

i

$HS(CH_2)_nCH_3$

$S—(CH_2)_nCH_3$

n = 6 (*n*), 7 (*o*)

Scheme 4. Synthesis of compounds to test the role of the substituent position on the E ring and of the distance between E ring and urea group

Dehydroprogesterone

HS–C_6H_4–$(CH_2)_nNH_2$

CH_3CH_2NCO

$(CH_2)_nNH_2$

SH, NH_2

NH_2

$(CH_2)_nNHCONHCH_2CH_3$

t (n=1), ***u*** (n=2)

CH_3CH_2NCO

NCO

$NHCONHCH_2CH_3$

NHCONH

p (meta), ***q*** (ortho)

r (meta), ***s*** (ortho)

Scheme 5. Synthesis of the carbon-bridged analog of PgA4(+)

$HOCH_2CH_2OH$, P-TsOH

O_2N–C_6H_4–CH_2MgBr, Cu(I), -78°C

$FeSO_4$, NH_4OH

(1) H^+, H_2O
(2) HPLC

NCO

CH_2 NHCONHCH

Carbon-bridged PGA4(+)

Table 1. Solubility of PgA4 in different solvents

Solvent	*Solubility (mg/ml)*
dH_2O	0.00179
Ethanol	30.4
DMSO	27.216
Propylene glycol	13.371
Glycerin	0.674
20% 2-hydroxypropyl-β-cyclodextrin (HPCD)	2.48

Table 2. Relative potency of different PgA compounds in reversing the MDR1 phenotype

Analog	"R" function	*Relative Potency*[a]	*Relative Potency (MDR1-specific)*[b]	*Rf_I/Rf_{II}*[c]
PgA4 (+)		**1**	**1**	0.200/0.560
PgA4(-)		0.91 (0.57/1.24)	0.80 (0.40/1.20)	0.200/0.560
PgA11	CH_3	0.74	0.58	0.244/0.592
PgA8		0.78 (0.82/0.74)	0.99/--[d]	0.248/0.608
PgA13		**2.36**	**3.1**	0.280/0.580
PgA20	CH_3	**2.30** (2.54/2.07)	**2.63** (3.05/2.21)	0.252/0.612
PgA28	CF_3	0.91	0.71	0.232/0.580
PgA12		1.26 (1.87/0.85)	1.12 (1.46/0.78)	0.236/0.588
PgA5	O_2 S	**0.33**	--[d]	0.080/0.300
PgA3	CH_3	0.69	0.80	0.100/0.400
PgA2	CH_2Cl	0.72	0.63	0.120/0.560
Pg	(Progesterone)	0.0242	--[d]	0.520/0.800
Vrp	(Verapamil)	0.36	0.23	

Legend to Table 2:

[a] Relative Potency = $EC_{50(PgAx)}/EC_{50(PgA4)}$ where mean observed $EC_{50(PgA4)}$ was 641.5+/-93.2 SE

[b] MDR1-specific Relative Potency = $sEC_{50(PgAx)}/sEC_{50(PgA4)}$, where each sEC_{50} represent the EC_{50} obtained by interpolation on the dose-response curve corrected by subtraction of the increase of doxorubicin accumulation caused by the PgA in MDR1-negative cells . The mean observed $sEC_{50(PgA4)}$ was 1000.7 +/- 74.5SE

[c] Rf_{I}= Retention Factor as calculated by TLC using Hexane:EtAc (1:1) as the mobile phase
Rf_{II}= Retention Factor as calculated by TLC using Hexane:EtAc (1:18) as the mobile phase

[d] The highest tested concentration does not reach the 50% reversal level after correction for non MDR1-specific effect

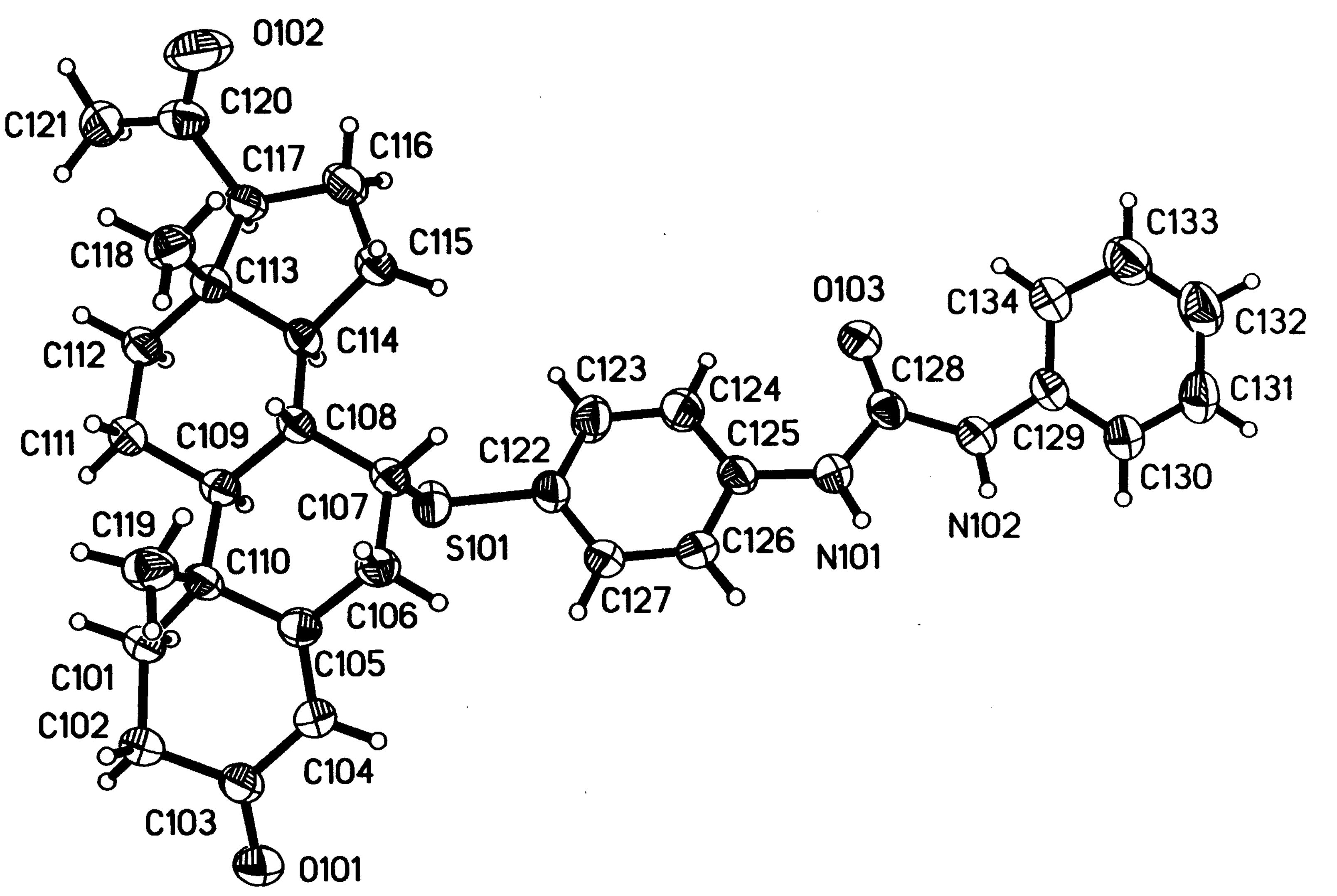

Figure 1. Ball and stick drawing of the X-ray crystal structure for compound PGA13.

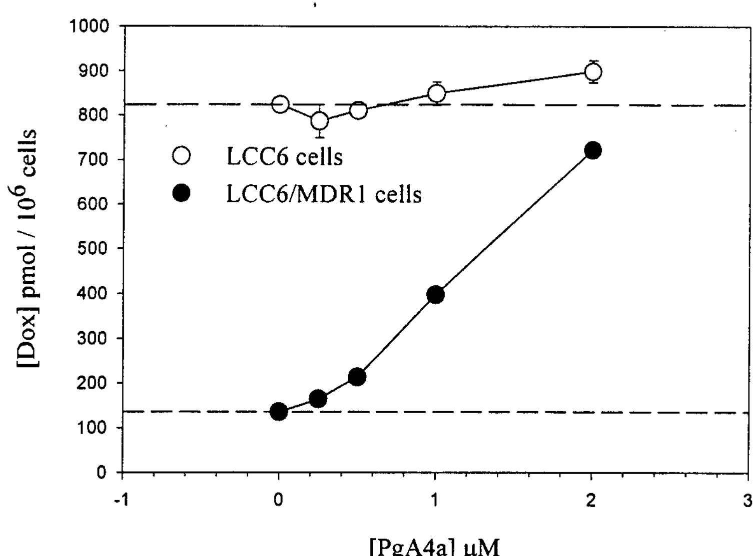

Figure 2. Doxorubicin accumulation in LCC6 and LCC6/MDR1 cells: effect of PgA4 treatment. Long dashed lines: doxorubicin accumulation in untreated LCC6 cells. Medium dashed lines: doxorubicin accumulation in untreated LCC6/MDR1 cells

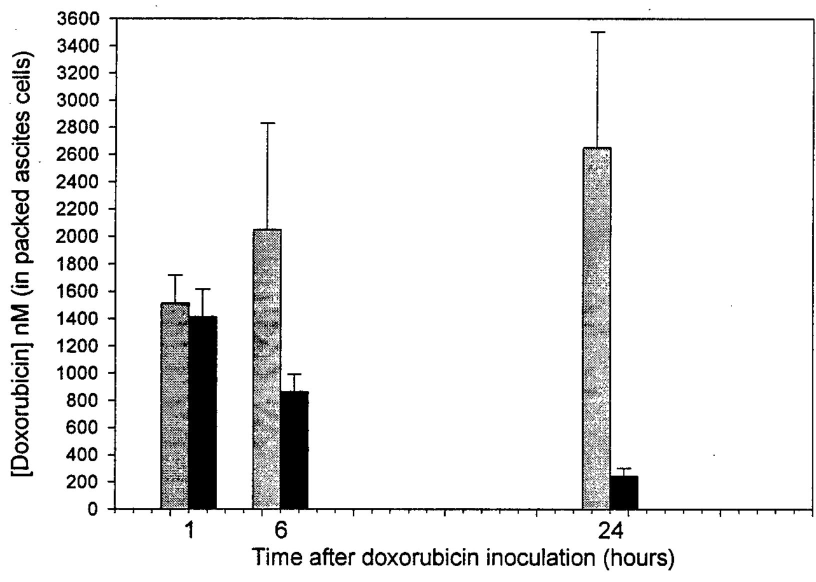

Fig. 3. Time dependence of doxorubicin accumulation in LCC6 and LCC6/MDR1 ascites cells *in vivo.*

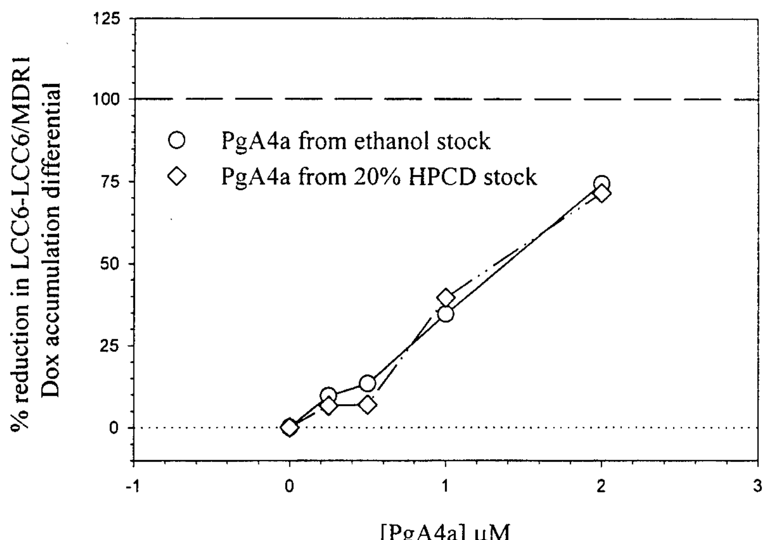

Fig. 4. Effect of PgA4 from ethanol and HPCD stock solutions on doxorubicin accumulation accumulation differential in LCC6/MDR1 cells. Long dashed lines: doxorubicin accumulation in untreated LCC6 cells (defined as 100% of the differential). Medium dashed lines: doxorubicin accumulation in untreated LCC6/MDR1 cells (defined as 0% of the differential).

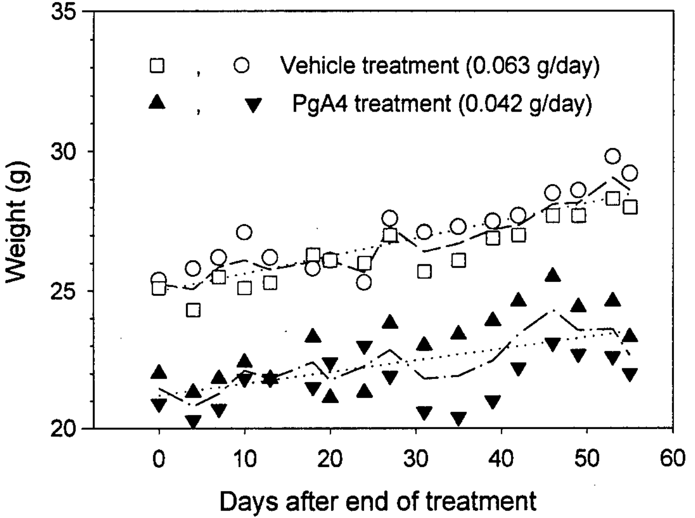

Fig. 5. Weight gain of Ncr *nu/nu* mice following treatment with PgA4. Mice were treated with PgA4 15.4 mg/Kg, twice per day, for 3 days. Each symbol represents values relative to a single mouse Dashed lines represent average values per treatment group.

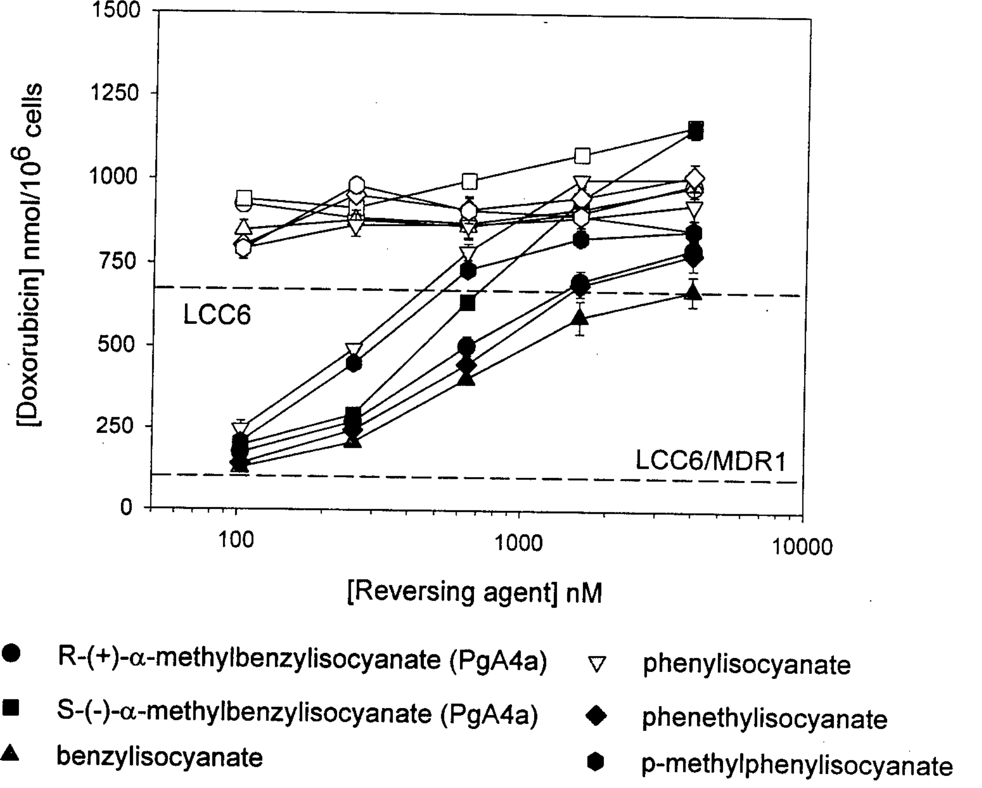

Fig. 6. Effect of some PgA4 compounds on doxorubicin accumulation in Pgp-positive (LCC6/MDR1) and –negative (LCC6) cells. Results of a representative experiment. Dashed lines represent accumulation in each cell line in the absence of PgA treatment.

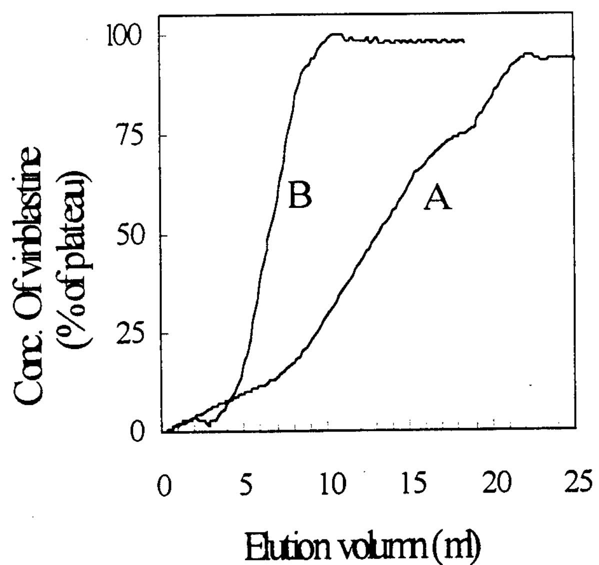

Fig .7. Frontal chromatography of VBL on the PGP-SP

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Irving W. Wainer	Professor

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
Wayne State University, Detroit, MI	B.S.	1965	Chemistry
Cornell University, Ithaca, NY	Ph.D	1970	Organic Chemistry
Institute of Molecular Biology, Eugene, OR	Postdoctoral	1970-73	Molecular Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Employment, Experience, Honors:

1973-78 Research Associate, Dept. of Pharmacology, **Thomas Jefferson University**, Philadelphia, PA

1978-86 Research Chemist (GS-13), Division of Drug Chemistry, **US FDA**

1986-90 Director of Analytical Chemistry, Clinical Pharmacokinetics Lab and Associate Member, Pharmaceutical Division, **St. Jude Children's Research Hospital**, Memphis, TN

1988-94 Editor-in-Chief, ***Chirality***

1990-97 Professor, Department of Oncology, **McGill University,** Montreal, Canada

1992- Recipient **"A J P Martin Medal"** presented by the **Chromatographic Society**

1993- Senior Editor, ***Journal of Chromatography B: Biomedical Applications***

1994 Elected Fellow**, American Academy of Pharmaceutical Sciences**

1995- Elected Member, **United States Pharmacopeial Convention Committee of Revision**

1997- Professor, Dept. of Pharmacology, **Georgetown University Medical Center**

1997- Head, Bioanalytical Center, **Georgetown University Medical Center**

Publications: In the past 3 years, I.W. Wainer has published 29 articles in refereed journals and 10 additional manuscripts have been accepted for publication, 3 books, 2 book chapters and 21 abstracts. Recent and previous publications relevant to this application are presented below:

Kaliszan, R., Noctor, T.A.G. and Wainer, I.W. Stereochemical aspects of benzodiazepine binding to human serum albumin II: Quantitative relationship between structure and enantioselective retention in high performance liquid affinity chromatography. *Molec. Pharmacol.,* 42:512-517, 1992

Noctor, T.A.G., Pham, C.D. Kaliszan, R and Wainer, I.W.: Stereochemical aspects of

benzodiazepine binding to human serum albumin I: Enantioselective high performance liquid affinity chromatographic examination of chiral and achiral binding interactions between 1,4-benzodiazepines and human serum albumin. *Molec.Pharmacol.*, 42:506-511, 1992
Noctor, T.A.G. and Wainer, I.W.:The use of displacement chromatograph to alter retention and enantioselectivity on a human serum albumin-based HPLC chiral stationary phase: A mini-review., *J.Liq.Chromatogr.*, 16:783-800, 1993

Kaliszan, R., Noctor, T.A.G. and Wainer, I.W.: Quantitative structure-enantioselective retention relationships for the chromatography of 1,4-benzodiazepines on a human serum albumin based HPLC Chiral stationary phase: An approach to the computational prediction of retention and enantioselectivity. *Chromatographia*, 33:546-550, 1993

Kaliszan R, Kaliszan, A and Wainer, I.W The prediction of drug binding to melanin using a melanin-based HPLC stationary phase and chemometric analysis of the chromatographic data. *J.Chromatogr.*, 666:221-234, 1994

Booth, T.D. and Wainer, I.W.: Investigation of the enantioselective separations of α-alkyl arylcarboxycylic acids on an amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase using quantitative structure-enantioselective retention relationships (QSERR): Identification of a conformationally driven chiral recognition mechanism. *J.Chromatogr.A*, 737:157-169, 1996.

Kaliszan, R. and Wainer, I.W.: Combination of biochromatography and chemometrics: A potential new research strategy in molecular pharmacology and drug design. *Chromatographic Separations Based On Molecular Recognition*, Jinno, K. (ed.), Wiley-VCH, New York, 1997, pp. 273-302.

Booth, T D., Azzaoui, K and Wainer, I.W.: Prediction of chiral chromatographic separations using combined multivariate regression and neural networks. *Analytical Chemistry*, 69:3879-3883, 1997

Azzaoui, K., Diaz-Perez, M.J., Zannis-Hadjoupoulos, M., Price, G.B., Wainer, I.W.: The effect of steroids on DNA synthesis in an in vitro replication system: Initial QSAR studies and construction of a non-estrogen receptor pharmacophore. J Med Chem, 41/9: 1392-1398, 1998

Pasternyk, M., Felix, G., Descorps, V., Ducharme, M.P. and Wainer, I.W.: On-line deconjugation of glucuronides using an immobilized enzyme reactor based upon β-glucuronidase. J. Chromatogr. B, 715:379-386, 1998.

Azzaoui, K., Diaz-Perez, M.J., Zannis-Hadjoupoulos, M., Price G.B., Wainer, I.W.: Receptor independent enhancement of DNA replication by steroids. J Cellular Biochem., in press

Sotolongo, V., Johnson, D.V., Wahnon, D, Wainer, I.W.: Immobilized horse liver alcohol dehydrogenase as an on-line high performance liquid chromatographic enzyme reactor for stereoselective synthesis. Chirality, in press

Pasternyk, M., Felix, G., Descorps, V., Ducharme, M.P. and Wainer, I.W.: On-line deconjugation of chloramphenicol-β-D-glucuronide on an immobilized β-glucuronidase column: Application to the direct analysis of urine samples. J. Chromatogr. A., in press

Zhang, Y., Xiao, Y., Keller, K.J. and Wainer, I.W.: Immobilized nicotinic receptor stationary phase for on-line liquid chromatographic determination of drug-receptor affinities. Anal. Biochem., in press

BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME	POSITION TITLE
Ji-Hyun Kim, Ph.D.	Postdoctoral Fellow

EDUCATION *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Ewha Women University, Seoul, ROK	B.S.	1992	Chemistry
Georgetown University, Washington, DC	Ph.D.	1998	Organic Synthesis
Georgetown University, Washington, DC	Postdoctoral	1998-date	Breast Cancer

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

Research and Professional Experience:

Jan-March 1990 Visiting Student at Kyoto University in Japan and Beijing University in China
1993-1998 Graduate Research Assistant, Georgetown University, Department of Chemistry
1998-date Postdoctoral Fellow, Lombardi Cancer Research Center, Georgetown University, Washington, DC

Other Committments of Time: No sabbatical or extended leave anticipated.

Proportion of Time to be Devoted to This and Other Research: **This application:** 100 %. **Other current/pending applications:** None.

Current or Prior Federal Government Service: None

Graduate Students: None.

Other Personnel: None:

Number pages consecutively at the bottom throughout the application. Do *not* use suffixes such as 3a, 3b.

Publications:

1. **Kim, J-H.**, Kulawiec, R. J. "Synthesis of 2-phenyl-2-cycloalkenones via palladium-catalyzed tandem epoxide isomerization-intramolecular aldol condensation" *Tetrahedron Lett.* **1998**, *39*, 3107-3110.

2 **Kim, J-H.**, Kulawiec, R. J. "A Tandem Epoxide Isomerization-Aldol Condensation Processed Catalyzed by Palladium Acetate-Tributylphosphine" *J. Org. Chem.* **1996**, *61*, 7656-7657.

Selected Abstracts:

1 **Kim, J-H.**, Kulawiec, R. J. "Palladium-mediated Enolization: a novel tandem epoxide isomerization-aldol condensation reaction" 210th National Meeting of the American Chemical Society – Abstr **ORGN 0151**, 1995.