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The goal of this project is to build upon our discovery of two phospholipid lead compounds, serine amide phosphate (SAP) and serine diamide phosphate (SDAP), that have been shown to be selective in their cytotoxic actions in PC-3 and DU-145 prostate cancer cells respectively. These agents were originally designed as part of a series of compounds to inhibit lysophosphatidic acid (LPA), a phospholipid growth factor. After discovering the antiproliferative activity of SAP and SDAP in prostate cancer cell lines we propose to synthesize a focused set of SAP and SDAP analogs. We have found that the synthesis of these compounds can be prepared in a shorter sequence and in better yield using our new synthetic scheme. We have tested for the affinity of the synthesized compounds in PC-3, DU-145, and LNCaP cell lines as we proposed earlier. In addition to these cell lines we have also tested for affinity of these compounds in two additional PPC-I and TSU cell lines (data shown in Table 1). These new analogs have provided valuable insight as to the importance of chirality, lipid solubility, spatial orientation, and important functional groups of the pharmacophore and for the optimization of the antiproliferative actions of this new set of drugs. Our most recent compounds are based on the thiazolidinones (2) and the thiazolidine (3) analogs. We have utilized new synthetic schemes for these new compounds and have found the optimum length of the aliphatic chain in these two series. In earlier studies it appeared in our Serine Amide Phosphate (SAP) series that the aliphatic chain is optimum at C-14 while with the new compounds it appears to be C-18 on DU-145 and PC-3 cell lines. In a few instances we have discovered a new set of 2-arylthiazolidine-4-carboxylic acid amides that show sub micromolar anticancer activity in the cell lines described above. We have designated this set of compounds as 2-arylthiazolidine-4-carboxylic acid amides (ATCAAs). This report shares the critical structure activity relationships for optimum activity in prostate cancer cells.
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Introduction The goal of this project is to build upon our discovery of two phospholipid lead compounds, serine amide phosphate (SAP) and serine diamide phosphate (SDAP), that have been shown to be selective in their cytotoxic actions in PC-3 and DU-145 prostate cancer cells respectively. These agents were originally designed as part of a series of compounds to inhibit lysophosphatidic acid (LPA), a phospholipid growth factor. After discovering the antiproliferation activity of SAP and SDAP in prostate cancer cell lines we propose to synthesize a focused set of SAP and SDAP analogs using the combinatorial parallel-compound solution phase syntheses when appropriate, and to prepare the remaining analogs using classical techniques. These analogs provided us with valuable insight as to the importance of chirality, lipid solubility, spatial orientation, and important functional groups of the pharmacophore and allow for the optimization of the antiproliferative actions of this set of drugs.

More recently we have discovered a new set of 2-arylthiazolidine-4-carboxylic acid amides that show sub micromolar anticancer activity in the cell lines described above. We have found new synthetic schemes for these new compounds and have expanded our structure activity relationships into the substitutions for activity against PC-3, DU-145, LNCaP, PPC-1 and TSU-Pr1 prostate cell lines using the RH7777 cell line as a control cell line for comparison. We are now optimizing these agents for potential use in prostate cancer.

Due to time and budgetary constraints, only a limited set of compounds have been carried forward. These experiments are designed to provide an initial pharmacologic assessment of our most promising compounds, focusing specifically on (1) their in vivo toxicity and (2) their in vivo antitumor efficacy in prostate tumor xenografts. Animal care guidelines at our institution will be strictly followed for these studies. We have found a new set of compounds, the 2-arylthiazolidine-4-carboxylic acid amides (ATCAAs) that have potential for prostate cancer.
Task 1. Synthesis of serine amide phosphate (SAP) and serine diamide phosphate (SDAP) analogs

Year 4: We will take the advantage of biological studies in year 1-3 to design new generation of analogs in order to optimize the inhibition of proliferation of prostate cancer cells.

This task was successfully completed. In year 2 we described design, synthesis, and biological evaluation of a new series of 2-aryl-4-oxothiazolin-3-yl amides in which 4-thiazolidine moiety was introduced as a phosphate mimic. However, these 4-thiazolidinone derivatives demonstrated less cytotoxicity in prostate cancer cells despite improved selectivity over RH7777 cells. To further optimize the thiazolidinone analogues in terms of cytotoxicity and selectivity, we made closely related structural modifications, which led us to the discovery of a new class of 2-arylthiazolidine-4-carboxylic acid amides (ATCAAs). The detailed structure activity relationship studies of this 3rd generation compounds was reported in year 3 report. These compounds were potent cytotoxic agents with IC_{50} values in the low micromolar concentration range and demonstrated enhanced selectivity in receptor-negative cells compared to serine amide phosphates (SAPs) and 4-thiazolidinone amides (manuscript 1).

Apoptosis represents a general and delicately efficient cellular suicide pathway. Most of the presently available cytotoxic anticancer drugs mediate their effect via apoptosis induction in cancer cells. Apoptosis is suggested as one of the major mechanisms for targeted therapy of various cancers including prostate cancer. However, cancer cells become resistant to apoptosis in case of advanced prostate cancer and do not respond to cytotoxic chemotherapeutic agents. Thus, agents that induce apoptotic death of hormone-refractory prostate cancer cells could be useful for the treatment of this malignancy.

Recently, we showed that ATCAAs induce apoptosis in LNCaP and PC-3 cells (manuscript 1). Therefore, we hypothesize that ATCAAs represent a novel class of anti-prostate cancer agents, which were very effective in the inhibition of growth of human prostate cancer cell lines and capable of inducing apoptosis. To further understand the structural features and their anticancer activity, we proposed synthetic optimization of ATCAAs toward potency and selectivity.
The details of this work have been recently published in Bioorganic & Medicinal Chemistry Letters (manuscript 2).

The general synthesis of various analogs is shown in Scheme 1. Accordingly, L-cysteine (1a) or L-penicillamine (1b) was allowed to react with appropriate benzaldehydes (2a-2e) in ethanol at ambient temperature to give cyclized products (3-7), which were converted to the corresponding Boc derivatives 8-12 as shown in Scheme 1. Reaction of Boc-protected carboxylic acids 8-12 with octadecyl or di-n-octyl amine using EDC/HOBt gave corresponding amides, which were treated with TFA to form the target compounds 13-18. All new compounds were fully characterized by $^1$H NMR, $^{13}$C NMR, IR and Mass spectrometry, in certain cases by elemental analysis.

Cancer-bearing animals have elevated levels of polyamines in their extracellular fluids. The potential usefulness of polyamine analogs as antiproliferative agents against many tumor cell lines has been extensively discussed. Prostate gland is a uniquely rich factory of polyamine production. The semen of healthy men contains large amounts of spermine that originates mainly from prostatic secretion. No other human organ has such high polyamine concentrations. Therefore, targeting prostatic polyamines has been a tempting approach for the therapy of prostatic carcinoma.

In year 2 we designed and synthesized serine-polyamine and thiazolidinone-polyamine conjugates. The antiproliferative effects of synthesized compounds were assessed against five human prostate cancer cell lines DU-145, PC-3, LNCaP, PPC-1, and TSU-Pr1 using sulforhodamine B (SRB) assay.
Interestingly, the thiazolidinone-spermine conjugate (19) showed enhanced selective antiproliferative activity in prostate cancer cell lines over non-tumor RH7777 cells. Encouraged with these results and in our continued efforts to further optimize this set of compounds for selective potency and to improve their pharmacokinetic properties, we designed a new series of compounds containing thiazolidine-4-carboxylic acid as head group conjugated with naturally occurring polyamines. Initially, we utilized putrescine, spermidine, and spermine polyamines for this study.

Carboxylic acid 22 was synthesized following a method, reported earlier from our laboratory. Treatment of excess of 1,4-diaminobutane with di-t-butyl-dicarbonate in chloroform under dilute conditions gave mono protected putrescine (24). Reaction of acrylonitrile with 1,4-diaminobutane in methanol gave the adduct which was converted to Boc-protected spermidine (26) in two steps as shown in Scheme 2. Reaction of carboxylic acid 22 with protected polyamines (24 and 26) in the presence of EDC/HOBt followed by treatment with HCl gave compounds 29 and 30 (Scheme 2).

Scheme 2

![Scheme 2 Diagram](image)

We adopted a different protocol for the synthesis of spermine conjugates. Firstly, carboxylic acid 22 was converted to corresponding active ester with 4-nitrophenol. Reaction of this active ester 31 with spermine in methanol at ambient temperature gave the corresponding spermine conjugate which was treated with HCl/Et₂O to form the compound 32 (Scheme 3).
Task 2. Determine activity of SAP and SDAP analogs in Prostate cell lines

Year 4: We will determine the activity of the synthesized analogs in PC-3, DU-145 and LNCaP cell lines.

This task was completed successfully. We have tested the cytotoxicity of the synthesized compounds in PC-3, DU-145 and LNCaP prostate cancer cell lines as proposed earlier. In addition to these cell lines we have also tested in two additional PPC-1 and TSU-Pr1 prostate cancer cell lines. To determine the selectivity of these compounds we have also tested them in non-prostate cancer cells RH7777 cells (data shown in Tables 1 & 2).
Table 1. Antiproliferative effects of synthesized analogs

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<td></td>
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<td>&gt;20</td>
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<tr>
<td>5-Fluorourasil</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.9</td>
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</tbody>
</table>

<sup>a</sup>Control cell line. <sup>b</sup>Prostate cancer cell lines. <sup>c</sup>ND = Not determined.
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<td>32. HCl</td>
<td><img src="image" alt="Structure" /></td>
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</table>

ᵃControl cell line. ᵇProstate cancer cell lines. ᶜBreast cancer cell line.
Task 3. Determine the activity of SAP and SDAP analogs in prostate tumor xenograft in mice

Year 4: We will select the most promising agents from specific Aim 6 of the PC-3, DU-145 and LNCaP cell lines studied in year 1, 2 and 3 for In Vitro Efficacy against Prostate Tumor Xenografts in mice (Specific Aim C.7)

Before assessing in vivo activities of these thiazolidine analogs (ATCAAs), we first tested the acute (30 day) toxicity and pharmacokinetics of selected thiazolidines. Daily subcutaneous doses (10 mg/kg of analog A, 5 mg/kg of analog B, 10 mg/kg of analog C, or 10 mg/kg of analog D) did not produce any signs of toxicity as demonstrated by lack of body weight loss. We developed and validated an LC/MS bioanalytical method for quantitation of drug concentrations in mouse plasma. Briefly, plasma proteins were precipitated with acetonitrile, centrifuged, and the supernatant fraction directly injected to the LC/MS. Analytes were detected using selected ion monitoring in the positive-ion mode using electrospray ionization and an Agilent 1100 coupled to a single quadrupole mass spectrometer.

We then examined the pharmacokinetics of analog A in ICR mice after a 10 mg/kg intravenous dose (Figure 1). Compound A demonstrated moderate clearance (56 mL/min/kg) and distribution (volume of distribution was 1.3 L/kg), with a terminal half-life of 2 h. The relatively short in vivo half-life of analog A in mice prompted us to examine the in vitro hepatic metabolism of several lead compounds (i.e., analogs A-D; Figure 2). Analog A with a shorter (C14) alkyl chain was metabolized more rapidly in vitro compared to B with a C18 alkyl chain. Thiazolidines C and D with 2-aryl ring substituents were most stable during in vitro metabolism studies. Hydroxylation of the alkyl chain was a major metabolic modification found in A, with the subsequent formation of a carboxylic acid metabolite, suggesting that alkyl chain length is also an
important determinant of in vivo metabolic stability and pharmacokinetics. Importantly, the structural modifications that enhanced in vitro cytotoxicity also enhanced in vitro metabolic stability.

**Figure 2. In Vitro Metabolism of Thiazolidines**

![Graph showing in vitro metabolism of thiazolidines](image)

**Key Research Accomplishments**

a) Investigated the effect of position of the substituents and nature of the ether linkage on phenyl head group of ATCAAs.

b) Optimized the substitution pattern of methoxy groups on the phenyl head group by synthesizing new analogs. Cytotoxicity data shows that 3,4,5-trimethoxyphenyl analog was more active than corresponding 4-methoxy, 3,4-dimethoxy, and 2,4,6-trimethoxyphenyl derivatives.

c) Investigated the significance of amide group in ATCAAs by replacing the amide hydrogen with an alkyl group to provide branched amide 16, which failed to demonstrate cytotoxicity at concentration below 20 μM in three prostate cancer cell lines except LNCaP and PPC-1 cells.

d) Observed that central thiazolidine core in ATCAAs with two chiral centers plays an important role in providing potency and selectivity as simple structural modification by dimethyl substitution at C-5 position lead to decreased potency in all five human prostate cancer cell lines.

e) Cytotoxicity data demonstrated that ATCAAs are sensitive to simple modifications or changes, which allowed us to understand the minimum structural requirements of this class
of compounds to exhibit potent and selective anticancer activity against prostate cancer cells.

f) Synthesized new series of polyamine conjugates containing thiazolidine-4-carboxylic acid as head group conjugated with naturally occurring polyamines. In this small series of compounds, spermine conjugates were found to be most active with enhanced selectivity against prostate cancer cell lines and interestingly they did not show any cytotoxicity in MCF-7 breast cancer cells below 100 μM.

g) Toxicity and pharmacokinetics of selected ATCAAs indicates that these compounds did not produce any signs of toxicity as demonstrated by lack of body weight loss.

h) We developed and validated an LC/MS bioanalytical method for quantitation of drug concentrations (ATCAAs) in mouse plasma.

i) Identified metabolic sites of selected thiazolidines by in vitro hepatic metabolism studies.

**Reportable Outcomes (Copies attached)**

1. Discovery of 2-Arylthiazolidine-4-carboxylic acid amides as a new class of cytotoxic agents for prostate cancer.

2. SAR studies of 2-arylthiazolidine-4-carboxylic acid amides: A novel class of cytotoxic agents for prostate cancer.

3. Polyamine Conjugates of Serine, 4-Thiazolidinone and Thiazolidine-4-carboxylic acid: Synthesis and Growth Inhibitory Effects on Human Prostate Cancer Cell Lines.
Discovery of 2-Arylthiazolidine-4-carboxylic Acid Amides as a New Class of Cytotoxic Agents for Prostate Cancer

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To improve the selectivity and antiproliferative activity of previously reported serine amide phosphates (SAPs), we designed a new series of 4-thiazolidinone amides, in which the 4-thiazolidinone moiety was introduced as a phosphate mimic. However, these 4-thiazolidinone derivatives demonstrated less cytotoxicity in prostate cancer cells despite improved selectivity over RH7777 cells. To further optimize the thiazolidinone analogues in terms of cytotoxicity and selectivity, we made closely related structural modifications, which led us to the discovery of a new class of 2-arylthiazolidine-4-carboxylic acid amides. These compounds were potent cytotoxic agents with IC$_{50}$ values in the low micromolar concentration range and demonstrated enhanced selectivity in receptor-negative cells compared to SAPs and 4-thiazolidinone amides.

Introduction

One promising drug development strategy for prostate cancer involves identifying and testing agents that interfere with growth factors and other molecules involved in the cancer cell’s signaling pathways. G-protein-coupled receptors (GPCRs) are a family of membrane-bound proteins that are involved in the proliferation and survival of prostate cancer cells initiated by binding of lysophospholipids (LPLs). The importance of G protein-dependent pathways in the regulation of growth and metastasis in vivo is corroborated by the observation that the growth of androgen-independent prostate cancer cells in mice is attenuated by treatment with pertussis toxin, an inhibitor of G/o proteins. Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (SIP) are lipid mediators generated via the regulated breakdown of membrane phospholipids that are known to stimulate GPCR-signaling.

LPLs bind to GPCRs encoded by the Edg gene family, collectively referred to as LPL receptors, to exert diverse biological effects. Lysosphatidic acid (LPA) stimulates phospholipase D activity and PC-3 prostate cell proliferation. Further, prior studies have shown that LPA is mitogenic in prostate cancer cells and that PC-3 and DU-145 cells express LPA$_1$, LPA$_2$, and LPA$_3$ receptors. Advanced prostate cancers express LPL receptors and depend on phosphatidylinositol 3-kinase (PI3K) signaling for growth and progression to androgen independence. Thus, these pathways are widely viewed as one of the most promising new approaches to cancer therapy and provide an especially novel approach to the treatment of advanced, androgen-refractory prostate cancer. Despite the promise of this approach, there are no clinically available therapies that selectively exploit or inhibit LPA or PI3K signaling.

In a previous contribution from our laboratory, we showed that effective cytotoxic agents were obtained, by replacing the glycerol backbone in LPA with serine amide. However, the most potent compounds in that series of derivatives were nonselective and potently killed both prostate cancer and control cell lines. To improve the selectivity and enhance the pharmacokinetic and antiproliferative properties, 2-aryl-4-oxo-thiazolidine amides with general structure III (Figure 1) were designed, utilizing 4-thiazolidinone moieties as a biomimetic replacement for the phosphate group. This strategic modification showed that the 2-arylthiazolidine moiety is indeed quite beneficial for obtaining a new set of antiproliferative compounds with improved selectivity, but resulted in decreased potency compared to serine amide phosphates. To further optimize the structural characteristics of these compounds to selectively elicit antiproliferative activity, we made closely related, minor modifications to 2-aryl-4-oxo-thiazolidine amides as shown in Figure 1. Our current work highlights synthesis, structure-activity relationship (SAR) studies, and biological evaluation of 2-arylthiazolidine-4-carboxylic acid amides (ATCAAs) for prostate cancer.

Figure 1.

APPENDIX 1


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The ability of 2-aryl-thiazolidine derivatives (ATCAAs) to inhibit the growth of five human prostate cancer cell lines (DU-145, PC-3, LNCaP, PPC-1, and TSU–Pr1) was assessed using the sulforhodamine B (SRB) assay. We also included a control cell line (RH7777) that does not express LPL receptors, to understand whether the antiproliferative activity of these derivatives was mediated through inhibition of LPL receptors. We first examined LPL receptor expres-
Table 2. LPL Receptor mRNA Expression

<table>
<thead>
<tr>
<th>LPL receptor</th>
<th>old name</th>
<th>RH7777</th>
<th>DU145</th>
<th>PC-3</th>
<th>LNCaP</th>
<th>PPC-1</th>
<th>TSU-Pr1</th>
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<td>LPA1</td>
<td>EDG-2</td>
<td>UD</td>
<td>2.16</td>
<td>2.53</td>
<td>UD</td>
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<td>LPA2</td>
<td>EDG-4</td>
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<tr>
<td>LPA3</td>
<td>EDG-7</td>
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<td>0.28</td>
<td>0.15</td>
<td>UD</td>
<td></td>
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</table>

sum LPA1-3    | 2.56    | 3.23   | 0.60  | 2.85 | 2.32  |

* UD: under detection limit.

Table 3. Antiproliferative Effects of Compounds 3-29 and 34

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<th>compd</th>
<th>RH7777</th>
<th>DU-145</th>
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<th>LNCaP</th>
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</table>

* Control cell line. * Prostate cancer cell lines. * No activity. * Not determined. * IC50 in nM.

The diastereomeric mixtures of the target compounds 3-29 were used as such to evaluate their in vitro inhibitory activity against prostate cancer cell lines, and the results are summarized in Table 3. Paclitaxel and 5-fluorouracil were used as reference drugs for comparison. Since preparation of isolated enantiomers was not easy to achieve, the IC50 values were obtained on diastereomeric mixtures in order to select the most promising compounds. Many of these thiazolidine analogues were very effective in killing prostate cancer cell lines with IC50 values as low as 480 nM (Table 3). Examination of the cytotoxic effects of 3-5 showed that as the chain length increased from C7 to C18, the potency also increased. However, a further increase in the alkyl chain length by one carbon unit (6) caused a significant loss of activity. Interestingly, the C14 derivative (4) demonstrated higher potency than 5, but was 8-fold less selective against the RH7777 cell line. Thus, an alkyl chain with C18 unit was optimal for maintaining the potency and selectivity observed in this series of compounds. N-Acylation and N-sulfonyl derivatives (28 and 29) were significantly less cytotoxic than parent compound 5. Replacement of the phenyl ring with an alkyl or cyclohexyl group reduced the potency (7 and 8) relative to the thiazolidine derivative (5). Introduction of a methylene spacer separating the phenyl ring and the thiazolidine ring furnished a compound 9, which was less active than the parent compound 5.

Replacements of the phenyl ring with a heterocycle, such as an indole, pyridine, or furan ring was investigated by synthesizing analogues 10-12. The furanyl derivative 12 showed equivalent cytotoxicity as 5, but was 3-fold less selective against RH7777 cells.

The cytotoxicity data of compounds 13-27 provides a summary of a broad survey of phenyl ring-substituted analogues. Examination of the IC50 values of these analogues demonstrates a greater tolerance for diverse substituents in the phenyl ring. In general, the most potent analogues possessed electron-donating substituents in the phenyl ring.
From this study, compound 18 emerged as one of the most potent and selective cytotoxic agents with an IC_{50} of 0.55 μM and 38-fold selectivity in PPC-1 cells. Further, the ability of these analogues to induce apoptosis in LNCaP and PC-3 cells provides an important clue to understand their mechanism of action, and suggests that they may have therapeutic utility in the treatment of prostate or ovarian cancer. All compounds discussed in this report have been prepared and tested as diastereomeric mixtures. Future efforts shall be aimed at synthesis and evaluation of pure individual stereoisomers of the most promising thiazolidines discussed above.

**Experimental Section**

All reagents and solvents used were reagent grade or were purified by standard methods before use. Moisture-sensitive reactions were carried out under an argon atmosphere. Progress of the reactions was followed by thin-layer chromatography (TLC) analysis. Flash column chromatography was carried out using silica gel (200–425 mesh) supplied by Fisher Scientific. Melting points were measured in open capillary tubes on a Thomas-Hoover melting point apparatus and are uncorrected. All compounds were characterized by NMR and MS (ESI). 1H NMR spectra were recorded on a Varian 300 instrument. Chemical shifts are reported as δ values relative to Me4Si as an internal standard. Mass spectra were obtained in the electrospray (ES) mode using Esquire-LC (Bruker) spectrometer.

**Table 4. Thiazolidine Amide-Induced Apoptosis**

<table>
<thead>
<tr>
<th>Compd for 72 h</th>
<th>PC-3</th>
<th>LNCaP</th>
<th>RH7777</th>
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<tr>
<td>2 μM</td>
<td>1.8</td>
<td>14.1</td>
<td>2.6</td>
</tr>
<tr>
<td>5 μM</td>
<td>18.7</td>
<td>75.4</td>
<td>3.2</td>
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</table>

*ND: not determined.*

...}

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (μM)</th>
<th>LNCaP</th>
<th>PC-3</th>
<th>RH7777</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a-v</td>
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2-Aryl-thiazolidine-4-carboxylic acid amides (ATCAAs) were obtained by the modification of previously reported 4-thiazolidinones. We synthesized a number of ATCAAs and evaluated for their inhibitory activity toward the growth of human prostate cancer cell lines. Introduction of ring activating groups on the phenyl ring resulted in increased potencies for prostate cancer cell lines and led to discovery of several new anticancer agents represented by analogues 16, 17, and 18 with low/sub micromolar cytotoxicity and high selectivity.

Conclusions

From this study, compound 18 emerged as one of the most potent and selective cytotoxic agents with an IC_{50} of 0.55 μM and 38-fold selectivity in PPC-1 cells. Further, the ability of these analogues to induce apoptosis in LNCaP and PC-3 cells provides an important clue to understand their mechanism of action, and suggests that they may have therapeutic utility in the treatment of prostate or ovarian cancer. All compounds discussed in this report have been prepared and tested as diastereomeric mixtures. Future efforts shall be aimed at synthesis and evaluation of pure individual stereoisomers of the most promising thiazolidines discussed above.
4.01 (t, J = 7.5 Hz, 0.6H), 3.83 (s, 3H), 3.39–3.55 (m, 1H), 3.18–3.26 (m, 1H); MS (ESI) m/z 220 (M+) + 1.

2-Phenylthiazole-4-carboxylic Acid Methyl Ester (32). This compound was synthesized following a reported procedure.\(^{14}\) N-Bromosuccinimide (2.48 g, 13.9 mmol) and benzyol peroxide (0.05 g) were added to 31 (1.5 g, 8.7 mmol) dissolved in CCl\(_4\) (70 mL), and the solution was refluxed for 6 h. Solvent was removed in vacuo, and the crude product was purified by column chromatography to afford 32 (0.71 g, 48%). \(^{13}\)H NMR (CDCl\(_3\)) \(\delta 8.10 \text{ (s, 1H)}, 7.96–7.93 \text{ (m, 2H)}, 7.46–7.50 \text{ (m, 3H)}, 3.49 \text{(dd, } J = 13.5, 6.9 \text{ Hz, 2H)}, 1.69 \text{ (m, 1H)}, 1.27 \text{ (m, 30H)}, 0.89 \text{(t, } J = 6.3 \text{ Hz, 3H}); MS (ESI) m/z 457.60 (M+ 1).

Cell Cultivation. DU-145, PC-3, and LNCaP human prostate cancer cell lines, and RH7777 rat hepatoma cells were obtained from American Type Culture Collection (Manassas, VA). Dr. Mitchell Steiner at University of Tennessee Health Science Center kindly provided PFC-1 and TSU–Pr1a cells. Prostate cancer cells and RH7777 cells were maintained in RPMI 1640 medium and DMEM (Mediatech, Inc., Herndon, VA), respectively, supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) in 5% CO\(_2\)-containing humidified atmosphere at 37 °C.

RT-PCR Analysis of LPA Receptor Expression. Total RNA was extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instruction. 0.5 \(\mu\)g (LPA\(_1\)) or 1 \(\mu\)g (LPA\(_2\) and LPA\(_3\)) of total RNA was used to perform RT-PCR using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen Corp., Carlsbad, CA) with 0.2 \(\mu\)M of primers. The following primer pairs were used: LPA\(_1\) forward 5'-GTCCTCAACAGGATGACACAC-3', reverse 5'-TGCGTTATTGTGTAGTAGTCGC-3'; LPA\(_2\) forward 5'-CTGCCACGGAGGCTTTCAGG-3', reverse 5'-AGACGACCCAGAAGT-GAAG-3'; LPA\(_3\) forward 5'-CCCAAGCTCTCAGCTGAA-3', reverse 5'-TTCCCTTGTAGAGATCATGAGGCGG-3'; \(\beta\)-actin forward 5'-GCTGCTGGCAGGCAACAACGTC-3', reverse 5'-CAACATGACCTGGTCTCTTTC-3'. PCR conditions were as follows: After 2 min denaturation step at 94 °C, samples were subjected to 34 to 40 cycles at 94 °C for 30 s, 60 °C (LPA\(_1\)) or 58 °C (LPA\(_2\) and LPA\(_3\)) for 30 s, and 72 °C for 1 min, followed by an additional elongation step at 72 °C for 7 min. Primers were selected to span at least one intron of the genomic sequence to detect genomic DNA contamination. The PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and the band intensity was quantified using Quantity One Software (Bio-Rad Laboratories, Inc., Hercules, CA). Expression levels of each receptor subtype in different cell lines were expressed as ratios compared to \(\beta\)-actin mRNA.\(^{12}\)

Cytotoxicity Assay. For in vitro cytotoxicity screening, 1000 to 5000 cells were plated into each well of 96-well plates depending on growth rate and exposed to different concentrations of a test compound for 96 h in three to five replicates. All the compounds were dissolved in dimethyl sulfoxide at 5 to 20 mM and diluted to final concentrations in complete culture medium. Cell numbers at the end of the drug treatment were measured by the SBR assay. Briefly, the cells were fixed with 10% of trichloroacetic acid and stained with 0.4% SBR, and the absorbances at 540 nm were measured using a plate reader (DYNEX Technologies, Chantilly, VA). Percentages of cell survival versus drug concentrations were plotted and the IC\(_{50}\) (concentration that inhibited cell growth by 50% of untreated control) values were obtained by nonlinear regression analysis using WinNonlin (Pharsight Corporation, Mountain View, CA). 5-Fluorouracil was used as a positive control to compare potencies of the new compounds.

Apoptosis. A sandwich ELISA (Roche, Mannheim, Germany) utilizing monoclonal antibodies specific for DNA and histones was used to quantify degree of apoptosis induced by the analogues after 72 h exposure. This assay measures DNA-histone complexes (mono- and oligonucleosomes) released into cytoplasm from the nucleus during apoptosis. RH7777 cells were employed because of nonspecific cytotoxicity of compound 4 in receptor-negative cells as well as receptor-positive prostate cancer cells.

Acknowledgment. This research was supported by a grant from the Department of Defense (DAMD17-01-1-083). Pharsight Corporation generously provided WinNonlin software through an Academic License.

Supporting Information Available: \(^{1}H\) NMR (300 MHz) and MS (ESI) characterization data for compounds 2b–v and 4–29 are available free of charge via the Internet at http://pubs.acs.org.

References

SAR studies of 2-arylthiazolidine-4-carboxylic acid amides: A novel class of cytotoxic agents for prostate cancer

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Abstract—In our continuing efforts to develop novel chemotherapeutic agents for prostate cancer, recently we reported the discovery of 2-arylthiazolidine-4-carboxylic acid amides (ATCAAs) as a new class of cytotoxic agents. Several of them were very effective in killing specific human prostate cancer cell lines with low/sub-micromolar cytotoxicity and high selectivity against control cells in our sulforhodamine B assay. Encouraged with these preliminary results, we decided to further optimize this new scaffold to enhance the potency and selectivity. Current work describes the synthesis, SAR, and biological evaluation of new compounds for their ability to inhibit the growth of five human prostate cancer cell lines. The cytotoxicity data demonstrated that ATCAAs are sensitive to simple modifications or changes, which allowed us to understand the minimum structural requirements of this class of compounds to exhibit potent and selective anticancer activity against prostate cancer cells.

Prostate cancer is the most common cancer and is the second leading cause of cancer-related deaths in North America.1 According to American Cancer Society, approximately 30,000 men will die from prostate cancer in the United States in 2005.2 One out of nine men over 65 years of age is frequently diagnosed with prostate cancer in the United States.3 Age and hormone are two known factors influencing the incidence of prostate cancer. Recently, dietary pattern has been identified as a major factor for the difference in prostate cancer incidence between Western and Asian countries.3 5 Hormonal ablation, the basis of systemic therapy, will inevitably fail to control the progression of metastatic prostate cancer in the long run.6 Patients with advanced or metastatic prostate cancer develop hormone-refractory status that becomes fatal because of the growth of androgen-independent tumor cells and the emergence of tumor clones. Agents that induce apoptosis in metastatic prostate cancer are necessary for the cancer chemotherapy and are urgent for the clinical treatment.

Keywords: Prostate cancer; GPCRs; Synthesis; SAR.

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Recent signal transduction research has raised the idea that intracellular signaling mechanisms triggered by extracellular hormonal factors acting through heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) can mediate and sustain prostate cancer pathologic process.7 Patients with advanced prostate cancer express elevated levels of GPCRs and GPCR ligands, suggesting that the GPCR system is activated in the cancerous gland and may contribute to tumor growth.8 Importantly, inhibition of G protein signaling attenuates prostate cancer cell growth in animal models.7 However, the nature of intracellular signaling pathways mediating mitogenic effects of GPCRs in prostate cancer is poorly defined.

Apoptosis represents a general and delicately efficient cellular suicide pathway. Most of the currently available cytotoxic anticancer drugs mediate their effect via apoptosis induction in cancer cells.9 Apoptosis is suggested as one of the major mechanisms for targeted therapy of various cancers including prostate cancer.10 12 However, cancer cells become resistant to apoptosis in case of advanced prostate cancer and do not respond to cytotoxic chemotherapeutic agents.13 Thus, agents that induce apoptotic death of hormone-refractory prostate cancer
The general synthesis of target compounds is shown in Scheme 1, against RH7777 cells. Comparison of the growth of human prostate cancer cell lines and capable inhibition of LPL receptors. To validate their use as negative controls, which were very effective in the inhibition of negative activity of ATCAAs was mediated through effects of ATCAAs was mediated through the GPCRs. Accumulating evidence suggests that LPA's actions are concordant with many of the hallmarks of cancer, indicating an important role for LPA in the initiation or progression of malignant disease. Indeed, LPA levels are significantly increased in malignant effusions, and its receptors (LPA1/2/3) are aberrantly expressed in prostate cancer cells. Further, we showed that ATCAAs induce apoptosis in LNCaP and PC-3 cells. Therefore, we hypothesize that ATCAAs represent a novel class of anti-prostate cancer agents, which were very effective in the inhibition of growth of human prostate cancer cell lines and capable of inducing apoptosis. To further understand the structural features and their anticancer activity, we herein propose synthetic optimization of ATCAAs toward potency and selectivity. In this paper, we report the synthesis, structure-activity relationship, and antiproliferative activity of new ATCAAs for prostate cancer.

The general synthesis of target compounds is shown in Scheme 1. Accordingly, L-cysteine (5a) or L-penicillamine (5b) was allowed to react with appropriate benzaldehydes (6a-6e) in ethanol at ambient temperature to give cyclized products (7-11), which were converted to the corresponding Boc derivatives 12-16 as shown in Scheme 1. Reaction of Boc-protected carboxylic acids 12-16 with octadecyl or di-n-octyl amine using EDC/HOBt gave corresponding amides, which were treated with TFA to form the target compounds 17-22. All new compounds were characterized by spectroscopy and, in certain cases, by elemental analysis. The structure and antiproliferative effects of synthesized compounds along with previously reported ATCAAs (for comparison) are listed in Table 1.

The prepared compounds were tested for their potency and selectivity against five human prostate cancer cell lines (DU-145, PC-3, LNCaP, PPC-1, and TSU-Prl) and RH7777 cells (control cell line) using the sulforhoda mine B assay according to a previously reported procedure. RH7777 cells are rat hepatoma cells that does not express LPL receptors. These cells were used as negative controls to understand whether the antiproliferative activity of ATCAAs was mediated through inhibition of LPL receptors. To validate their use as negative controls, we also examined LPL receptor expression in these cells and showed that none of the LPL receptors were expressed in RH7777 cells by RT-PCR. 5-Fluorouracil (5-FU) was used as a reference drug. Analog 17 containing 4-hydroxyphenyl head group was equally active in all five prostate cancer cell lines, but was not selective compared to 1 (with 3-hydroxyphenyl group) against RH7777 cells. Comparison of the IC50 values of 2 and 18 suggests that an increase in the alkyl chain length of the ether leads to decreased cytotoxicity. Examination of the cytotoxicity data of ATCAAs suggests that electron-donating substituents on the 2-phenyl ring increase the biological activity, and compound 3 with 3,4,5-trimethoxyphenyl head group emerged as one of the most potent and selective cytotoxic agents from our previous study. It was also observed that 3,4,5-trimethoxyphenyl analog was more active than 3,4-dimethoxy and 4-methoxyphenyl derivatives. To further optimize the substitution pattern of methoxy groups on the phenyl ring, 19 was synthesized which showed a decrease in the potency compared to 3 in all prostate cancer cell lines.

![Figure 1](image_url)

![Scheme 1](image_url)
We showed that ATCAAs have demonstrated chain length (lipophilic side chain)-dependent cytotoxicity with shorter alkyl chain length containing compounds being less active. However, the effect of branching in the lipophilic tail region of ATCAAs on the biological activity was not examined before. To investigate the significance of amide group in ATCAAs, we decided to replace the amide hydrogen with an alkyl group. For these two reasons, compound 20 was synthesized and tested against five human prostate cancer cell lines. Analog 20 failed to demonstrate cytotoxicity at concentration below 20 μM in three prostate cancer cell lines except LNCaP and PPC-1 cells. Central thiazolidine core in ATCAAs with two chiral centers plays an important role in providing potency and selectivity. We observed that replacement of the thiazolidine ring with more stable thiazole ring resulted in loss of cytotoxicity. Compounds 21 and 22 were prepared to further optimize the central thiazolidine core by dimethyl substitution at C-5 position. However, this simple structural modification did not improve the activity. Indeed, 21 and 22 were active only above 20 μM against all tested five human prostate cancer cell lines.

Table 1. Antiproliferative effects of ATCAAs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC_{50} (μM)</th>
<th>RH7777</th>
<th>DU-143</th>
<th>PC-3</th>
<th>LNCaP</th>
<th>PPC-1</th>
<th>TSU-Pr1</th>
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</tr>
</tbody>
</table>

*S-FU | ND | 11.9 | 12.0 | 4.9 | 6.4 | 3.6 |

*a* Control cell line.

*b* Prostate cancer cell lines.

*c* ATCAAs for comparison.

*d* ND, not determined.
In conclusion, 2-arylthiazolidine-4-carboxylic acid amides represent a new class of cytotoxic agents for prostate cancer. Furthermore, the anticancer activity of these analogs is attributed to their ability to induce apoptosis in prostate cancer cells. In our continued efforts to optimize ATCAAs toward potency and selectivity, we have prepared and evaluated a new set of compounds for their ability to inhibit the growth of five human prostate cancer cell lines. The SAR study revealed that (1) antiproliferative activity of ATCAAs is sensitive to the position of the substituents on the phenyl ring, (2) introduction of dialkyl (i.e., dioctyl) amide group into the tail region decreases the potency, and (3) modifications to the central thiazolidine core are not favorable. The present data combined with our earlier SAR results provided an insight into the important structural requirements of ATCAAs for their anti-prostate cancer activity. On the basis of these results, we conclude that our next focus will be to incorporate these results, we conclude that our next focus will be to integrate into the important structural requirements of ATCAAs for their anti-prostate cancer activity. On the basis of these results, we conclude that our next focus will be to avons of ATCAAs 1 and their pharmacological characterization in animal models, the results of which will be reported in due course.

Acknowledgment

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References and notes

18. Characteristic data for some compounds are given below. Compound 18: 'H NMR (300 MHz, CDCl3) δ: 0.89 (t, J = 6.6 Hz, 3H), 1.27 (s, 32H), 1.44-1.43 (m, 3H), 3.29-3.34 (m, 2H), 3.38-3.41 (m, 1H), 3.71 (dd, J = 0.89 (t, J = 4.6 Hz, 3H), 1.26 (s, 32H), 3.12-3.45 (m, 3H), 3.72 (dd, J = 7.5, 4.5 Hz, 1H), 3.82 (d, J = 2.1 Hz, 3H), 3.84 (s, 6H), 4.14 (br s, 1H), 4.32-4.36 (m, 1H), 5.34 (d, J = 6 Hz, 1H), 5.86 (d, J = 7.5 Hz, 1H), 6.16 (d, J = 3.9 Hz, 2H), 7.38 (m, 1H), 13C NMR (75 MHz, DMSO-d6): δ: 13.8, 14.4, 22.0, 26.3, 28.6, 28.8, 28.9, 29.0, 31.2, 37.0, 62.8, 65.6, 66.1, 70.6, 71.6, 113.7, 113.9, 128.1, 158.0, 158.3, 169.6, 170.2; MS (ESI) m/z 505 [M+H]. Compound 19: 'H NMR (300 MHz, CDCl3) δ: 0.89 (t, J = 6.6 Hz, 3H), 1.26 (s, 32H), 3.12-3.45 (m, 3H), 3.72 (dd, J = 7.5, 4.5 Hz, 1H), 3.82 (d, J = 2.1 Hz, 3H), 3.84 (s, 6H), 4.14 (br s, 1H), 4.34 (d, J = 6 Hz, 1H), 5.54 (d, J = 7.5 Hz, 1H), 6.16 (d, J = 3.9 Hz, 2H), 7.38 (m, 1H), 13C NMR (75 MHz, DMSO-d6): δ: 13.8, 22.0, 26.1, 26.2, 28.6, 28.9, 31.2, 35.8, 55.2, 55.9, 62.5, 63.8, 66.0, 66.5, 91.5, 105.7, 105.9, 106.7, 169.6, 170.3; MS (ESI) m/z 511 [M+H]. Compound 20: 'H NMR (300 MHz, CDCl3) δ: 0.89 (t, J = 6 H, 3H), 1.27 (s, 32H), 1.46 (s, 3H), 1.50 (s, 3H), 2.97 (s, 6H), 3.19-3.30 (m, 2H), 3.58 (s, 0.6H), 3.95 (s, J = 0.4H), 5.54 (s, 0.5H), 5.64 (s, 0.5H), 6.25 (t, J = 6 Hz, 1H), 6.79 (dd, J = 8.7, 1.8 Hz, 2H), 7.38-7.46 (m, 2H); 13C NMR (75 MHz, CDCl3) δ: 13.5, 22.1, 25.5, 26.4, 27.2, 27.7, 28.7, 28.8, 28.9, 29.0, 29.1, 31.3, 38.4, 38.7, 39.9, 67.2, 67.9, 73.3, 74.2, 111.8, 111.9, 127.6, 127.9, 168.0, 169.6; MS (ESI) m/z 533 [M+H].
Polyamine Conjugates of Serine, 4-Thiazolidinone, and Thiazolidine-4-carboxylic Acid: Synthesis and Growth Inhibitory Effects on Human Prostate Cancer Cell Lines

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Abstract

We showed that arsenic trioxide phosphates (APAs), derivatives of thiazolidinone (TZA), represent a class of cytotoxic compounds that are effective and potent in killing prostate cancer cells. Although many of these compounds showed significant cytotoxicity, they were not selective. To improve the selectivity and antiproliferative activity of APAs, we designed a new series of 4-thiazolidinone analogues, in which the 4-thiazolidinone moiety was introduced as a phosphate mimic. However, these 4-thiazolidinone derivatives demonstrated less cytotoxicity in prostate cancer cells despite improved selectivity over normal cells. Further optimization of the thiazolidinone pharmacophore in terms of cytotoxicity and selectivity led us to the discovery of a third-generation 4-thiazolidinone-4-carboxylic acid amides. These compounds were potent cytotoxic agents with IC50 values in the low nanomolar concentration range and demonstrated enhanced selectivity in receptor-negative cancer cells compared to APAs and 4-thiazolidinones. During the course of structure-activity relationship (SAR) studies of above class of compounds, we were interested in investigating the effect of tetrazoles on the lipophilic amide side chain on potency and selectivity. It was also shown that polymerization of compounds extended a number of biological activities and have been utilized as therapeutic agents. Due to these reasons, we designed and prepared a series of compounds containing amide, 4-thiazolidinone and thiazolidine-4-carboxylic acid as head groups conjugated with naturally occurring polyamines like spermine, spermidine and spermine. Short chain polyamine conjugates to arsenic trioxides, thiazolidinones, and Thiazolidine-4-carboxylic acid did not show activity up to 100 μM. As the length of polyamine moiety increased, cytotoxicity also increased in prostate cancer cells. One of the polyamine compounds tested demonstrated selective cytotoxicity against prostate cancer cells, but not in breast or ovarian cancer cells. Their syntheses and biological studies will be presented in this presentation.

Introduction

Prostate cancer is the most common malignancy affecting men and is the second-leading cause of cancer deaths in the US. None of the conventional approaches to cancer therapy have proven to be highly effective for prostate cancer treatment. Advanced prostate cancers express androgen receptors (ARs) and depend on phytoestrogen-mediated androgen signaling for growth and progression to androgen-independent. Thus, these pathways are widely viewed as one of the most promising new approaches to cancer therapy. Androgen receptor signaling is critical for the progression of prostate cancer. It is the major regulator of prostate cancer cell survival, proliferation, and growth. The development of new androgen receptor antagonists is a major focus of research in prostate cancer. We hypothesized that replacement of the lipophilic amide side chain with polyamines improves water solubility of the class of drugs.

Figure 1

Summary

Conjunktion of serine, 4-thiazolidinone, and thiazolidine-4-carboxylic acid groups with naturally occurring polyamines provided a new series of cytotoxic compounds to prostate cancer. All synthesized compounds were evaluated for their ability to inhibit the growth of human prostate cancer cell lines. To determine their selectivity, lipid phases were tested in NCI-607 (LPA receptor negative), CV-I (normal), and MCF-7 (breast cancer) cells. Short chain polyamines conjugates with putrescine and spermidine did not show any cytotoxicity below 100 μM. As the length of the polyamine moiety increased (spermine), activity also increased in prostate cancer cells. Spermine conjugates (15 μM) with serine and 4-thiazolidinone head groups were more active than parent compounds (5 μM). Examination of cytotoxicity data of compounds 18 and 21 shows that polyamine conjugation increases selectivity over normal cells (NCT0077). Compounds 18 and 21 also demonstrated selective cytotoxicity (20-50% IC50) for prostate cancer cells over breast cancer cells.

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References