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Synthesis and Investigation of Novel Spiro-isoxazolines as Anti-Cancer Agents

Prasanta Das^a, Ann O. Omollo^a, Lungile J. Sitole^a, Eric McClendon^a, Edward J. Valente^b, Drazen Raucher^c, Leslie R. Walker^c, and Ashton T. Hamme II^{a,*}

^aDepartment of Chemistry & Biochemistry, Jackson State University 1400 J. R. Lynch St, PO Box 17910, Jackson, Ms 39217, USA

^bDepartment of Chemistry, University of Portland, Portland, OR 97203, USA

^cDepartment of Biochemistry, University of Mississippi Medical Center, Jackson, MS, 39216, USA

Abstract

A series of structurally diverse 4-bromo spiro-isoxazolines possessing a variety of aromatic and aliphatic substituents at the 3 position, were synthesized through a 1,3-dipolar cycloaddition followed by intramolecular cyclization of a pendant hydroxyl or carboxylic acid group. The biochemical antiproliferative activity was evaluated *in vitro* by using two breast cancer cell lines (MCF-7 and MDA-MB-231) and two prostate cancer cell lines (PC-3 and DU-145) using the MTT viability assay, and the IC₅₀ values were obtained. Spiro-isoxazoline derivatives bearing a *p*-chloro or an *o*-dichloro aromatic substituent at the 3-position of the isoxazoline showed considerable antitumor activities in all four cell lines with IC₅₀ value ranging from 43μM to 56μM.

Keywords

1,3-dipolar Cycloaddition; Stereoselective Intramolecular Cyclization; Regioselectivity; Spiro-isoxazolines; Bioevaluation

The World Health Organization (WHO) recently predicted that death caused by cancer is expected to increase to 13.1 million by 2030.¹ Among all other deadly cancers, invasive breast cancer is the most recurrently diagnosed malignant tumor among women² (31% in the US and 27% in Europe) and the second leading cause of cancer related death subsequent to lung cancer. Women have a 32% lifetime risk of developing breast cancer and 15% risk of breast cancer causing death in the US.³ The cause of breast cancer can be linked to a number

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*Corresponding author: Tel.: +1-601-979-3713; fax: +1-601-979-3674; ashton.t.hamme@jsums.edu (Ashton T. Hamme II).

Supplementary Material

Supplementary data (compound data including ¹H NMR, ¹³C NMR, IR, HRMS and X-ray) associated with this article can be found, in the online version, at www.sciencedirect.com.

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of risk factors that include early menarche, late menopause, nulliparous advanced maternal age, obesity, and genetic predisposition. Other factors that are thought to lead to breast cancer include high lipid diets, moderate alcohol consumption, and the use of exogenous estrogens.⁴ Likewise; prostate cancer also remains the next leading cause of cancer related death in men in the United States.⁵ Chemotherapy, one of the leading cancer therapy over other existing approaches such as surgery, radiotherapy, and targeted therapy has been extensively utilized for last few decades.⁶ However, the indiscriminate cytotoxicity of most of the chemotherapeutic agents between cancer and normal cell often engenders various frequent side effects (stroke, hair loss, weakness, bone and back pain, blood clots, fatigue etc.).⁷

These and other risk factors warrant the investigation of potentially new non-surgical techniques that can either arrest the growth or kill breast cancer cells. The most frequently used pharmaceuticals for cancer treatment are natural products or their synthetic analogues.^{8,9} Natural and nonnatural isoxazolines and spirocyclic isoxazoline derivatives have emerged as promising drug candidates due to their numerous biological activities¹⁰ resulting in an overall antiproliferative effect on a variety of cancer cell lines. The natural product 11-deoxyfistularin-3¹¹ contains two spirocyclic isoxazoline moieties and is cytotoxic toward estrogen dependent MCF-7 breast cancer cells ($LD_{50}=17$ mg/L). Other closely related natural products such as psammaplysin A,¹² aerothionin,¹³ homoaerothionin,¹³ fistularin-3¹⁴ and 11-ketofistularin-3¹⁵ also have spirocyclic isoxazolines as well as physiological activities. However the therapeutic value associated with the paucity in natural occurrence of biologically active molecules have been a long term inspiration for designing, synthesizing and investigating the biological activity of new molecules for the discovery and development of novel pharmaceuticals.

Considering the need for new structural moieties as novel pharmaceuticals and our ongoing interest in the isoxazoles and spiro-isoxazolines,^{16,17} we have previously developed common spiro-isoxazoline scaffolds retaining a wide range of structure modifications of biological interest. While our first variation is to introduce a range of aromatic substituent on the 3 position of the isoxazole ring, the second modification involves the introduction of cyclic ether and lactone spiro-linkages to the isoxazolines with varying ring size.

Herein, we have diversified our proposed scope by introducing several aromatic substitutions bearing electron donating and withdrawing groups to investigate different modes of interaction with cancer cell lines. Additionally we are also reporting the introduction of the ester functionality on the isoxazoline in order to investigate its comparative structure activity relationship with the aforementioned aromatic substituents. The activities of the spiro-isoxazoline derivatives were evaluated against the proliferation of various cancer cell lines that included breast [MCF-7(ER+), MDA-MB-231(ER-, PR-, HER2-)], and prostate [PC-3, and DU-145 (both AR- and PSA-)]. As part of our biological study, we used the MTT assay to evaluate their antiproliferative activity against the aforesaid cancer cell lines. Initially, cell viability studies were performed for screening the active compounds for further evaluation of IC_{50} values. To our delight, five compounds showed cytotoxic activity in both breast and prostate cancer cell lines (Fig. 2). The outcomes

of this study broaden the knowledge base for the structure activity relationships for this class of neoplastic inhibitors.

A series of spiro-furan, pyran and lactone based aromatic and ester substituted spiro-isoxazolines were developed. As shown in the retrosynthetic analysis in Scheme 1, alkynols, alkynoic acids will serve as dipolarophiles, while the 1,3-dipoles will be generated *in situ* to afford the corresponding isoxazole. Pyridinium tribromide (PTB) mediated intramolecular cyclization will then afford the cyclic ether based spiro-heterocycle, or the lactone containing spiro-isoxazoline.¹⁶

As shown by the results compiled in Scheme 2, isoxazoles **2(a–h)** were synthesized regioselectively by a base-promoted reaction of *in situ* generated 1,3-dipoles from corresponding hydroximoyl chlorides and alkynols **1(a,b)**.

Subsequently, pyridinium tribromide promoted¹⁶ spiro-etherification of the corresponding isoxazoles **2(a–h)** afforded the desired spiro-isoxazolines **3(a–h)** stereoselectively in moderate to good isolated yields (Scheme 2).

To widen the scope of our strategy, we have explored the synthesis of spiro-isoxazolines with an inherent lactone ring system. In this context, alkynoic acids **4a** and **4b** were subjected to 1,3-dipolar cycloaddition with an assortment of *in situ* generated aromatic nitrile oxides to afford compounds **5(a–h)** as shown in Scheme 3. Following the parallel protocol for spiro-etherification,¹⁶ smooth spirolactonization of isoxazoles **5(a–h)** occurred to provide the desired substituted spiro-isoxazolines **6(a–h)** as a single diastereomer in moderate to good yields (Scheme 3).

After accomplishing the syntheses of several spiro-isoxazoline systems as shown in Scheme 2 and 3, we then introduced ester functionality at the 3 position of isoxazoline moiety to extend the investigation of the structure-activity relationship of our compounds. Hence, we hypothesized that the reaction of an ester bearing 1,3-dipole precursor (e.g., ethyl nitroacetate or ethyl chloroximidoacetate) with the desired alkyne would allow access to the corresponding isoxazoles with a pendent alcohol and carboxylic acid which would undergo further intramolecular cyclization to furnish the spiro-moiety (Scheme 4) following the aforementioned pathway described for the syntheses of compounds **3(a–h)** and **6(a–h)** in Scheme 2 and 3. We observed that exposure of ethyl chloroximidoacetate to our optimal reaction conditions for 1,3-dipolar cycloaddition with alkynes furnished the furoxan as a major product, while the formation of desired isoxazoles **8(a–d)** were diminished. Despite this modest success with dehydrohalogenation of ethyl chloroximidoacetate, we decided to synthesize the desired isoxazole by dehydration of ethyl nitroacetate¹⁸ through an alternative base-promoted 1,3-dipolar cycloaddition as shown in Scheme 1. Subsequently we found the desired cycloadduct formation upon exposure of alkyne **7(a–d)** to ethyl nitroacetate in the presence of a catalytic amount of DABCO (Scheme 4).¹⁸ The purpose of the ester functionality is also for the future development of a library of novel molecules through an amide linkage for biological intervention studies. However, herein we are highlighting only two types of alkyl sources in order to compare its activity with the aforementioned molecules.

Having in hand the optimal reaction conditions for ethyl nitroacetate mediated 1,3-dipolar cycloaddition, we next explored the scope of our methodology by synthesizing isoxazoles using alcohol and carboxylic acid containing alkynes. Based upon the compilation of compounds in Scheme 4, a wide range of isoxazoles **8(a–d)** were employed for bromonium ion mediated spiro-cyclization. However, the spiro-etherification of ester substituted isoxazoles **8a** and **8c** with pendent alcohols did not proceed as anticipated. In this specific case we unexpectedly obtained a product which while not fully characterized appeared to result from an oxidative dimerization. In order to circumvent the unexpected reactivity of **8a** and **8c**, other brominating reagents and reaction conditions were investigated, including PTB/CH₂Cl₂-reflux, Br₂/THF-H₂O, Br₂/NaOAc/HOAc, and NBS/molecular sieves in the dark, to no avail. We finally realized that Br₂/CCl₄ in the absence of light preferentially produced the desired furan and pyran based spiro-isoxazolines (**9a** and **9c**) in good isolated yields (Scheme 4). In contrast, isoxazoles **8b** and **8d** with pendant carboxylic acids smoothly formed the corresponding spiro-lactones (**9b** and **9d**) through the original PTB reaction conditions in moderate to good isolated yields (Scheme 4). While the PTB/K₂CO₃ condition was suitable for the synthesis of **9b** and **9d**, other reaction conditions such as Br₂/CCl₄-dark were also found to be successful; however the presence of a weak base, such as K₂CO₃, appears to be indispensable reaction facilitators for **8b** and **8d**.

Finally, the crystalline nature and X-ray crystallographic analysis¹⁹ of compound **3c** and **9b** allowed for us to determine the stereochemical orientation of bromine and the spirocyclic oxygen (ether and lactone) which showed an *anti* relationship to each other (Fig. 1). Based upon a proposed mechanism describing the formation of **3a** and **6a** in our earlier report, we now expect the formation of compounds **9(a–d)** would also follow the analogous mechanistic pathways.¹⁶

Cell Viability Assay

After synthesizing the diverse array of spiro-isoxazolines, cell viability assays were performed in order to determine baseline activity levels for our compounds. In doing so, the synthetic spiro-isoxazolines (**3a–h**, **6a–h** and **9a–d**) analogues were tested *in vitro* against well-established cancer cell lines, two of them being the breast cancer cell lines MCF-7(ER +), MDA-MB-231(ER–, PR–, HER2–), and the other two being the prostate cancer cell lines PC-3, and DU-145 (both AR- and PSA-) to evaluate the spiro-isoxazoline's efficacy as antiproliferative agents. The individual cancer cells were treated with 100 μM concentration of each compound in duplicate for three days. The overall spectrum of cell viability assay indicated that a few of these spiro-isoxazolines can serve as a novel antiproliferative agent against all four cell line (Fig. 2). As depicted in Fig. 2, the corresponding spiro-isoxazoline derivative of *o*-dichloro furan **3d**, *p*-chloro pyran **3g**, *o*-dichloro pyran **3h**, *o*-dichloro spiro-lactone **6d** and compound **6e** exhibited promising activity in comparison to other spiro-isoxazolines. This study also reveals that the presence of spiro-furan, pyran, and lactone rings probably play an important role in their activity.

Having determined the active compounds with antiproliferative properties, we then established the IC₅₀ values through a MTT assay where the targeted cells were treated with the indicated compounds in quadruplicate at 20, 40 and 60 μM. Based upon the results

summarized in Table 1, compound **3g** demonstrates the most potent antitumor effect toward MCF-7, MDA-MB-231, PC-3, and DU-145, with respective IC₅₀ values of 51.2, 53.1, 56.7 and 48.1 μ M, whereas compound **3h** exhibited its most prominent anti-cancer activity toward MCF-7, MDA-MB-231, with IC₅₀ values of 52.4 and 43.1 μ M respectively (Table 1). Precise IC₅₀ values were determined only for compounds that exhibited activity below 60 μ M.

In summary, this article highlights the utility of the 1,3-dipolar cycloaddition as a tool to synthesize a number of synthetic intermediates for spiro-isoxazolines *via* an intramolecular cyclization of either alcohol or carboxylic acid precursors. We have synthesized several spiro-isoxazolines with a number of aromatic as well as ester substituents in the 3 position of the isoxazoline ring. Furthermore, we investigated the ability of a number of spiro-isoxazolines to inhibit the proliferation of both breast and prostate cancer cell lines. The data indicate that the spiro-isoxazolines can hinder cell proliferation regardless of receptor status. MCF-7 cells are estrogen receptor positive while MDA-MB-231 cells are negative for estrogen receptors, progesterone receptors, and are HER2/neu negative. The indicated compounds inhibited the growth of both cell lines similarly. Also indicative of being receptor independent, two prostate cancer cell lines were used, and the compounds were shown to have inhibitory effects. PC3 cells, which are androgen receptor negative, do not express prostate specific antigen, and are considered highly metastatic, and DU-145 cells, which are also AR and PSA negative and considered moderately metastatic, were both similarly affected by five compounds. While further studies are needed to determine mechanism of these compounds, i.e. what they are targeting, these studies prove that the spiro-isoxazolines have the potential to serve as a potent inhibitor of cancer cell growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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19. CCDC 970417 and CCDC 970416 contain the supplementary crystallographic data for compound 3c and 9b These data can be obtained free of charge via <http://www.ccdc.cam.ac.uk/conts/retrieving.html> (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; Fax: +44 1223 336033; E-mail: deposit@ccdc.cam.ac.uk)”.

Figure 1.
Thermal ellipsoid plots of **3c** and **9b**.

Figure 2.
Cell viability assay.

Scheme 1.
Retro synthetic analysis.

Scheme 2. Synthesis of furan and pyran based spiro-isoxazolines **3(a-h)**

Scheme 3. Synthesis of lactone containing spiro-isoxazolines **6(a-h)**.

Scheme 4. Synthesis of ester containing spiro-isoxazolines **9(a-d)**.

Table 1IC₅₀ values of compounds **3g** and **3h**.

Compounds	IC ₅₀ (37 °C) (μM ± SEM)			
	MCF-7	MDA-MB-231	PC-3	DU-145
3g	51.2 ± 0.85	53.1 ± 0.64	56.7 ± 1.23	48.1 ± 0.85
3h	52.4 ± 0.52	43.1 ± 0.96	*	*