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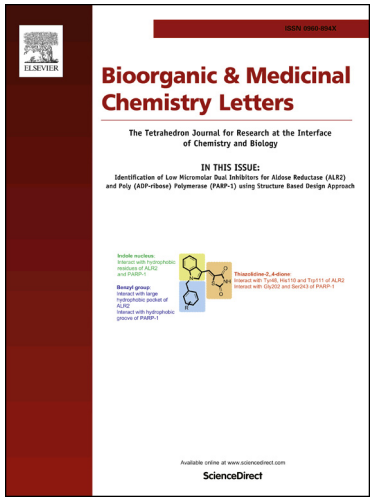

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PII: S0960-894X(17)30527-9
DOI: http://dx.doi.org/10.1016/j.bmcl.2017.05.046
Reference: BMCL 24991

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 3 April 2017
Revised Date: 9 May 2017
Accepted Date: 13 May 2017

Please cite this article as: Badolato, M., Carullo, G., Armentano, B., Panza, S., Malivindi, R., Aiello, F., Synthesis and anti-proliferative activity of a small library of 7-substituted 5*H*-pyrrole [1,2-a] [3,1] benzoxazin-5-one derivatives, *Bioorganic & Medicinal Chemistry Letters* (2017), doi: http://dx.doi.org/10.1016/j.bmcl.2017.05.046

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Synthesis and anti-proliferative activity of a small library of 7-substituted 5*H*-pyrrole [1,2-a] [3,1] benzoxazin-5-one derivatives

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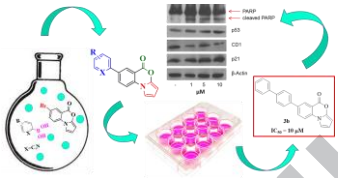

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Synthesis and anti-proliferative activity of a small library of 7-substituted 5*H*-pyrrole [1,2-a] [3,1] benzoxazin-5-one derivatives

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
benzoxazin-5-one
anti-proliferative activity
Suzuki-Miyaura cross coupling
Cell cycle analysis
Cancer cell lines

ABSTRACT

In this study, we investigate the anti-proliferative activity of a small library of 7-substituted 5*H*-pyrrolo[1, 2-a][3, 1]benzoxazin-5-one derivatives, against a panel of human cancer cell lines. We reported the synthesis of these compounds in a previous work. 7-bromo-5*H*-benzo[*d*]pyrrolo[2,1-b][1,3]oxazin-5-one showed a promising anti-proliferative effect. As starting material for Suzuki-Miyaura cross coupling reaction, it was selected for the design and the synthesis of six further derivatives, with the aim to better define structure-activity relationships. The anti-proliferative MTT assay revealed a dose-dependent reduction of cell viability, especially for 7-([1,1'-biphenyl]-4-yl)-5*H*-benzo[*d*]pyrrolo[2,1-b][1,3]oxazin-5-one. Cell cycle and western blotting analysis suggested apoptosis as possible mechanism for its anti-proliferative activity. These preliminary results encourage our interest for further optimizations.

Heterocycles containing benzoxazinone skeleton have been always attracting scaffolds in the field of medicinal chemistry (Fig. 1).

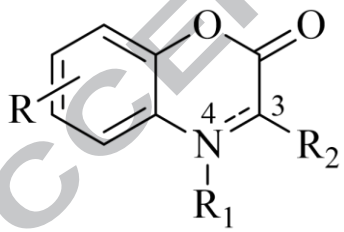


Figure 1. General structure of heterocycle containing benzoxazinone skeleton

Since their discovery as secondary metabolites of the grasses over 50 years ago, benzoxazinoids have been extensively studied. Different aspects of their chemistry and a wide range of their diverse biological activities have been published. Originally, they were found playing an important role in the chemical defense of plants, acting as natural pesticides and exhibiting allelopathic properties.[1,2] They also could function as genotoxins for human cells.[3] Inhibiting the bacterial type IIa topoisomerase, a series of 6-substituted benzoxazinone derivatives showed a rapid bactericidal activity.[4] Furthermore, benzoxazinoids showed thrombin and cyclooxygenase inhibitory properties, as well as antimicrobial and anti-inflammatory activities.[5,6]

Moreover, it has been demonstrated that benzoxazinoids could be useful for the treatment of cardiovascular diseases and cancer.[7,8]

The benzoxazinone skeleton contains three different potential areas for functionalization, the carbon atom in position 3 (C3), the nitrogen in position 4 (N4) and the aromatic ring. One of the modification that involved both the C3 and N4 atoms was their introducing in a fused pyrrole ring. A number of methods have been reported for the synthesis of 4*H*-pyrrolo[2,1-*c*][1,4]benzoxazin-4-one frame.[9,10]

Two derivatives of this class of compounds were identified as selective antagonists of G-protein coupled estrogen receptor (GPER), inhibiting the proliferation of SkBr3 cells and the migration of cancer-associated fibroblasts (CAFs) induced by 17β-estradiol and G-1.[11] Thus, heterocycles containing benzopyrroloxazinone frame can be considered as privileged scaffolds for the development of potential new drugs, including the treatment of cancer. The isomer 5*H*-pyrrolo[1,2-*a*][3,1]benzoxazin-5-one is not very studied yet. Recently, we reported the synthesis of a small library of 5*H*-pyrrolo[1,2-*a*][3,1]benzoxazine-5-one derivatives.[12]

In this study, our interest was to investigate about the anti-proliferative activity of those compounds against different human breast cancer cell lines with the aim to further develop an understanding of the SAR study of this class of compounds. From a preliminary screening, 5*H*-naphtho[2,3-*d*]pyrrolo[2,1-*b*][1,3]oxazin-5-one (**2**), and 7-bromo-5*H*-benzo[*d*]pyrrolo[2,1-

b][1,3]oxazin-5-one (**3**), bearing a bromine atom on C7, showed a good anti-proliferative activity against all used cell lines. On the contrary, the unsubstituted 5*H*-pyrrolo[1,2-*a*][3,1]benzoxazin-5-one (**1**) and 5*H*-pyrido[2,3-*d*]pyrrolo[2,1-*b*][1,3]oxazin-5-one (**4**) didn't show the same effect. Since compound **4** moderately inhibited the growth of the used cancer cells, the presence of benzene ring in the derivatives may be necessary for their anti-proliferative activity. Furthermore, the presence of substituents on the aromatic ring of compound **1** may improve the cytotoxicity of this class of compounds (Fig. 2).

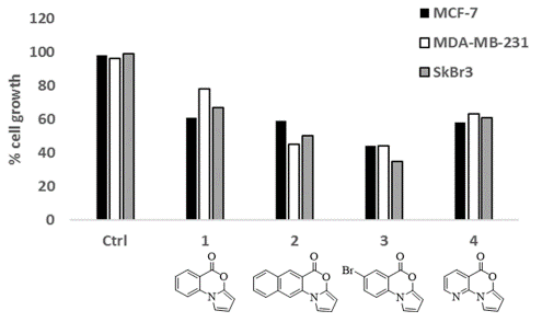


Figure 2. Some of 5*H*-pyrrolo[1,2-*a*][3,1]benzoxazin-5-one analogs previously prepared and their respective cytotoxicity at 50 μM

Continuously to our interest in the synthesis of 5*H*-pyrrolo[1,2-*a*][3,1]benzoxazin-5-one derivatives, having biological and pharmacological activities, we designed and synthesized a new series of 7-subsituted analogs and their *in vitro* anti-proliferative activity was evaluated, with the aim to better define the SAR, determining which moieties are essential for the anti-proliferative effect (Fig. 3).

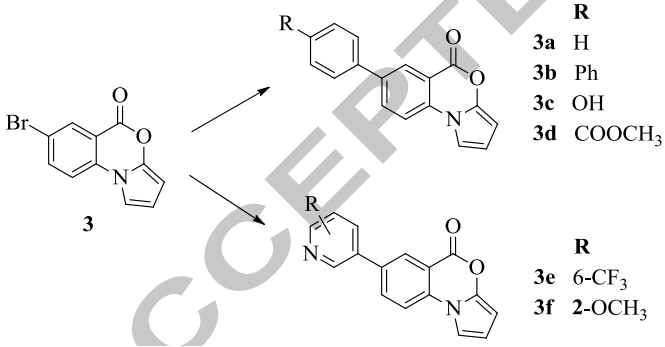
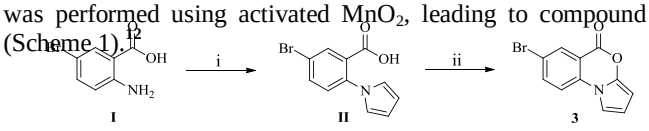


Figure 3. Compounds of interest to further investigate the SAR of 7-substituted 5*H*-pyrrolo[1,2-*a*][3,1]benzoxazine-5-one analogs

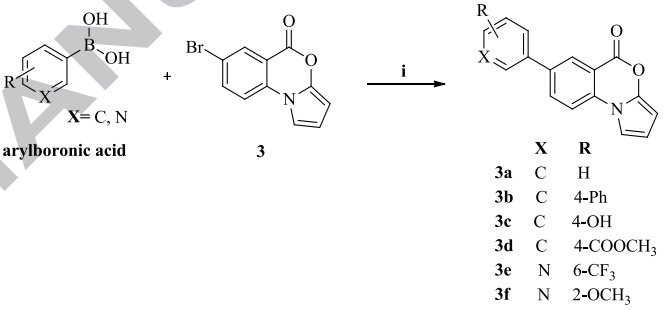
Due to its promising anti-proliferative activity, compound **3** was chosen as starting material, suitable substrate for coupling reaction. In particular, we performed Suzuki-Miyaura cross coupling, adjusting the reaction conditions for the synthesis of our derivatives. [13] To validate the importance of the substitutions on the aromatic ring, variously substituted phenyl, phenol and pyridines moieties were selected to decorate the new compounds. Then, a preliminary cytotoxicity was evaluated and the possible mechanism of action of this class of compounds was investigated.

Firstly, 5-bromo-2-(1*H*-pyrrol-1-yl)benzoic acid (**II**) was prepared by the reaction of **I** with 4-chloropyridine hydrochloride and 2,5-dimethoxytetrahydrofuran. The subsequent cyclization was performed using activated MnO_2, leading to compound **3** (Scheme 1).



Scheme 1. Reagents and conditions: (i) 4-Chloropyridine hydrochloride (1 equiv.), 2,5-dimethoxytetrahydrofuran (1.2 equiv.), 1,4-dioxane, reflux, 24 h; (ii) MnO_2 (5 equiv.), dry toluene, reflux, 24 h, **3** 30%.

Compounds **3a - 3f** were obtained in good yields and with a fast purification procedure, through crystallization, using a similar approach (Scheme 2). We adjusted the Suzuki-Miyaura cross coupling reaction, to optimize the synthesis of our derivatives. Since all the reactions were performed in water, TBAB was added to improve the solubility of the reactants and arylboronic acids were used as alcoholic solution. The quick reaction lasted about four hours and led to appreciable quantities of all the derivatives, except in the case of compound **3f**, due to the poor solubility of the starting (2-methoxypyridin-3-yl)boronic acid in the alcoholic solution and in others organic media.



Scheme 2. Reagents and conditions: (i) alcoholic solution of arylboronic acid (1.5 equiv.), **3** (1 equiv.), PPh_3 (0.3 equiv.), $Pd(OAc)_2$ (0.1 equiv.), TBAB (0.1 equiv.), 1M Na_2CO_3 (0.2 mL), water, 130°C, 4 h, **3a** 60%, **3b** 80%, **3c** 86%, **3d** 51%, **3e** 30%, **3f** 5%.

To establish the biological profile of all compounds, cell viability and proliferation were evaluated in MCF-7, MDA-MB 231, and SkBr3 breast cancer cell lines. To evaluate the possible toxicity of the derivatives, a mammary epithelial cell line (MCF-1

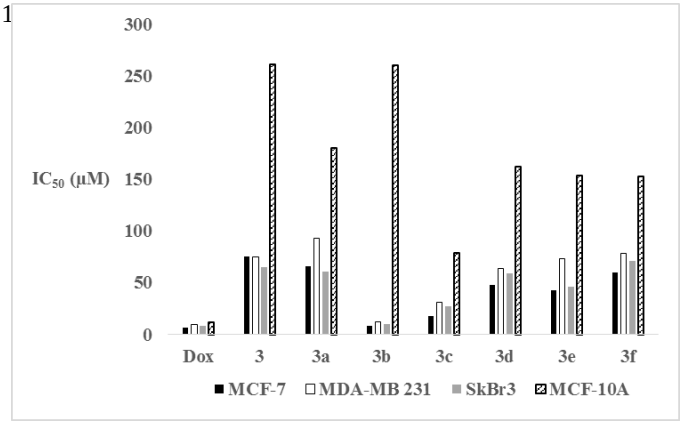


Figure 4. Biological profile of the synthesized derivatives; the titled compound **dox** is Doxorubicin, which was used as positive control.

As shown in Figure 4, most of the new synthesized compounds demonstrated a moderately improved anti-proliferative effect compared to the parent compound **3**. The presence of the unsubstituted phenyl ring in **3a** and the ethanone moiety in *para* in **3d**, did not affect the cell viability prominently. Compounds **3e** and **3f**, both presenting a pyridine ring, reflect the same behavior of the previous molecules. Compound **3c**, with a hydroxyl group in *para* position of the phenyl ring, showed a good anti-proliferative effect, and in all used cancer cell lines. However, it also demonstrated a cytotoxic activity against MCF-10A cells. On the contrary, compound **3b**, bearing a biphenyl ring able to confer a total planarity to the molecule, showed the best biological profile. It is interesting to note that, the biphenyl derivative, although demonstrated to be the most cytotoxic against the human cancer cells, it also was the safest compound against the mammary epithelial cells, with an IC_{50} value over 200 μM.

The mechanisms by which drugs induce death of cancer cells are different and include necrosis, apoptosis and autophagy. Therefore, an understanding of these mechanisms could be helpful to discover more convenient therapeutic strategies. To further examine the effect of compound **3b** on cell proliferation and survival, we analyzed the cell cycle phase distribution by flow-cytometric analysis of propidium iodide-stained cells after treatment with the compound. Figure 5 clearly displays that 24 h treatment with **3b** reduced the percentage of cells in the S-phase of the cell cycle, compared with control, in a dose-dependent manner. During the S-phase of the cell cycle, the genetic information of the DNA is synthesized and duplicated.

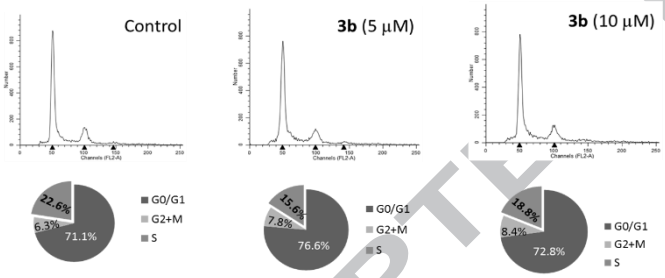


Figure 5. Cell cycle phase distribution in control and treatments samples.

Furthermore, the proteolysis of Poly (ADP-ribose) polymerase (PARP), crucial target indicating the presence of DNA damage and facilitating DNA repair, was estimated by immunoblotting analysis (Fig. 6). Increased levels of the proteolytic form of PARP (86 kDa) were detected in MCF-7 cells after 24 h treatment with compound **3b**, compared to the control. Increasing evidence has suggested the straightforward role of p53 signaling in both growth inhibition and apoptotic cascades.[14,15] p53 acts as a tumor suppressor depending on its physical and functional interaction with diverse cellular proteins, including some nuclear receptors that, in turn, exert an inhibitory activity on p53 biological outcomes. Activation of p53 by UV damage or other agents/signals results in p53-mediated transcription or up-regulation of genes such as the cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ to induce the apoptotic process, inhibiting the growth of cells with damaged DNA or cancer cells.[16-18]

The ability of compound **3b** to modulate the expression of p53,[19,20] along with its natural target gene $p21^{WAF1/Cip1}$ was also examined. The treatment with compound **3b** induced a significant increase in both p53 and $p21^{WAF1/Cip1}$ protein content in MCF-7 cells. Finally, changes in the expression of genes involved in cell cycle, such as cyclin D1 (CD1), were detected. CD1 is a critical modulator in the cycle G1/S transition and its overexpression is one of the most commonly observed alterations in human endometrial cancer. Thus, the potential ability of compound **3b** to modulate CD1 level in MCF-7 breast cancer cell line was evaluated.

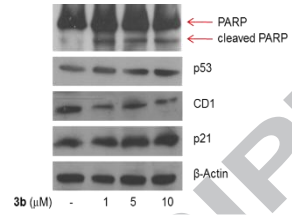


Figure 6. Immunoblots of PARP cleavage, p53, CD1, $p21^{WAF1/Cip1}$, from extracts of MCF-7 cells, treated with vehicle (ctrl), or compound **3b** (1, 5, 10 μM) for 24 hr. β-Actin was used as loading control.

Based on these promising results, further *in vitro* studies are ongoing, to evaluate the exact mode of action of selected compound **3b**. Additional decorations in the 5*H*-pyrrolo [1,2-*a*][3,1]benzoxazin-5-one structure have been performed in our laboratory, to obtain derivatives with better biological activity and lend them useful anticancer agents.

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A. Supplementary Material

- 5*H*-pyrrolo [1, 2-a][3, 1] benzoxazin-5-one is an interesting scaffold
- Various decorations have been performed to find an anti-proliferative agents
- Preliminary studies identified **3b** a good candidate
- The mechanism of action will be investigated