Bioorganic & Medicinal Chemistry Letters 28 (2018) 2789–2793

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

# Synthesis and evaluation of C2 functionalized analogs of the α-tubulin-binding natural product pironetin

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ARTICLE INFO

*Article history:*
Received 25 August 2017
Revised 21 October 2017
Accepted 24 October 2017
Available online 11 November 2017

*Keywords:*
Pironetin
Synthesis
Tubulin
Cytotoxicity
Structure-activity

ABSTRACT

Pironetin is an α-tubulin-binding natural product with potent antiproliferative activity against several cancer cell lines that inhibits cell division by forming a covalent adduct with α-tubulin via a Michael addition into the natural product's α,β-unsaturated lactone. We designed and prepared analogs carrying electron-withdrawing groups at the α-position (C2) of the α,β-unsaturated lactone with the goal to generate potent and selective binding analogs. We prepared derivatives containing halogens, a phenyl, and a methyl group at the C2 position to evaluate the structure-activity relationship at this position. Testing of the analogs in ovarian cancer cell lines demonstrated 100–1000-fold decreased antiproliferative activity.


## Main text

Pironetin (**1**, Fig. 1), a natural product isolated in 1993[1] and 1994,[2] has potent antiproliferative activity and acts via disruption of tubulin polymerization dynamics by binding to α-tubulin.[3–7] This mechanism differs from FDA-approved chemotherapeutics, which disrupt tubulin polymerization by binding to β-tubulin.[8–10] X-ray crystallography revealed that the natural product forms a covalent adduct with cysteine 316 in α-tubulin via Michael addition into the α,β-unsaturated lactone.[11,12] While pironetin has a unique mechanism of action, it has not been developed into a drug candidate. An in vivo study of pironetin showed poor efficacy and mice dosed with pironetin exhibited severe weight loss.[13] Since the in vivo toxicity could be due to non-selective formation of covalent adducts between pironetin and other biomolecules, we hypothesized that pironetin analogs that covalently label α-tubulin but form reversible bonds with off-target proteins could possess decreased off-target toxicity. Some support for this hypothesis was obtained, when we incubated pironetin the with monoethyl ester of glutathione and observed a glutathione-pironetin covalent adduct by LC–MS/MS analysis (see SI).

Previous studies reported that the addition of electron-withdrawing groups at the α-position of Michael acceptors can decrease off-target covalent adduct formation of covalent inhibitors. Taunton and coworkers showed that α-nitrile containing Michael acceptors form reversible covalent adducts with thiols and that "specific non-covalent interactions in concert with the covalent bond are needed to stabilize the complex" between the protein and the Michael acceptor for irreversible bond formation.[14,15] Using this principle, they were able to obtain a potent and selective MSK/RSK-family kinase inhibitor. In related work they showned that Michael acceptors containing electron-deficient aromatic groups and heterocycles at the α-position form reversible covalent bonds with thiols.[16] In a separate study, Yu and coworkers evaluated the effect of adding a fluorine to the EGFR-TK covalent inhibitor afatinib.[17] They found that a chemically tuned analog, containing a fluorine at the α-position of afatinib's α,β-unsaturated amide, was highly potent and had reduced off-target reactivity.

We therefore designed and prepared pironetin analogs with different functional groups at the α-position of the α,β-unsaturated lactone to evaluate structure-activity relationships and determine the feasibility of modifying the α-position of the natural product to decrease its non-selective covalent adduct formation. While our group along with other groups had previously evaluated the structure-activity relationship of different positions of the α,β-unsaturated lactone of pironetin and related analogs,[4,5,18–22] the effect of the addition of a functional group to the α-position of the α,β-unsaturated lactone has not been reported in the literature.

For the synthesis of the α-functionalized analogs **2**, we envisioned that the α,β-unsaturated lactone could be formed via lactonization of intermediate **3** (Scheme 1). The α,β-unsaturated lactone of pironetin has been synthesized using this method in several total syntheses.[23–29] The tri-substituted olefin could be

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https://doi.org/10.1016/j.bmcl.2017.10.057
0960-894X/

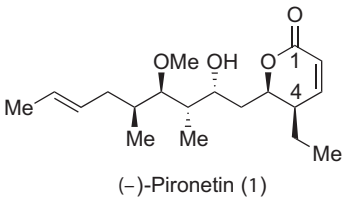



**Fig. 1.** Structure of pironetin.

synthesized via a selective olefination reaction of aldehyde **4**. A variety of olefination conditions have been reported for the synthesis of tri-substituted olefins carrying an alkoxide, halide, or alkyl moiety at the α-position.[30–35] An intermediate such as **4** could be synthesized from aldehyde **5**, which has been employed in previous syntheses of pironetin analogs in our group.[18]

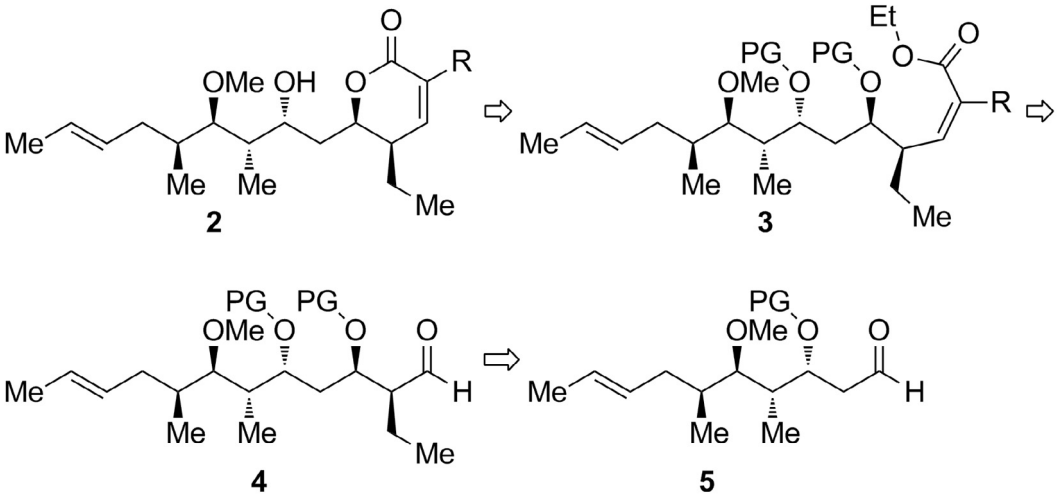
The synthesis of analogs **2** began with the diastereoselective aldol reaction between aldehyde **6** and thiazolidinethione **7** under conditions similar to those reported by Crimmins[23] and Marco[20] for the synthesis of pironetin and related analogs (Scheme 2). Following protection of the secondary alcohol as the TBS ether, the chiral auxiliary was cleaved with DIBAL-H to provide aldehyde **10**. We performed the desired olefination of aldehyde **10** with fluorine containing phosphonate ester **11a**, because the selective olefination with aryl phosphonate esters containing functional groups at the 2-position has been reported previously.[30,33] The olefination between aldehyde **10** and phosphonate ester **11a** proceeded in 63% yield to provide intermediate **12a**. We subsequently carried out the olefination reaction with the methyl, chlorine, and bromine containing phosphonate esters **11b**–**11d** to evaluate the SAR at the α-position of pironetin. While the olefination with 2-methyl phosphonate ester **11b** proceeded in high yield, we observed incomplete conversion for reactions with halogen-containing phosphonate esters **11c** and **11d**. We completed the synthesis of analogs **2** following the deprotection and lactonization of intermediates **12** under acidic conditions.

We also sought to synthesize analogs containing an aryl group at this position, since Michael acceptors containing electron-deficient aromatic groups at the α-position have been reported to be reversible covalent inhibitors.[16] While we proposed a series of analogs containing different aromatic groups at the α-position via a Suzuki coupling with α-bromopironetin analog **2d**, we initially synthesized α-phenyl analog **13** to determine if an aromatic group would be tolerated at the α-position (Scheme 3). Prior to the Suzuki coupling, the secondary alcohol in α-bromopironetin analog **2d** was protected as the silyl ether. The coupling between vinyl halide **14** and phenylboronic acid proceeded in moderate yield under mild conditions to give intermediate **15**. We completed the synthesis of analog **13** following removal of the TBS protecting group with $BF_3{\cdot}Et_2O$.

The antiproliferative activities of the new analogs were evaluated in drug-sensitive OVCAR5 and A2780 ovarian cancer cell lines (Table 1). The addition of any group at the α-position of pironetin resulted in decreased antiproliferative activity. The addition of a methyl group to the α-position of pironetin (entry 4) resulted in an approximate 200-fold decrease in activity. The α-chloro and α-fluoro analogs (entries 3 and 5) showed similar $GI_{50}$ values even though these halogens along with the methyl group have different electronic properties. The α-phenyl analog **13** was found to be inactive (entry 7). These results suggest that the decreased activity of the α-functionalized analogs could be due to the steric properties of the group at the α-position instead of the electronic properties of the various groups.

Although the α-functionalized pironetin analogs exhibited decreased biological activity relative to the natural product, α-bromopironetin **2d** had unique activity (entry 6). In the OVCAR5 cell lines, the dose response curve for α-bromopironetin **2d** showed biphasic character (Fig. 2). For this biphasic curve, the first inflection point occurs at a concentration approximately 3-times the $GI_{50}$ of pironetin (Table 1 entry 6); while the second point occurs at double-digit micromolar concentrations. However, a biphasic dose response curve was not observed when A2780 cell lines with treated with analog **2d** or with pironetin or the other analogs (Fig. 2). Another interesting aspect for the dose-response curves of α-bromopironetin **2d**, was the percentage of cells remaining at the high drug concentrations. In the dose-response curves of tubulin-binding agents, paclitaxel, pironetin and related analogs in the A2780 cell line, the dose-response curve plateaus at approximately 10–20% of the control population at the higher tested drug concentrations. In the dose response curves of α-bromopironetin **2d** in the A2780 cell line, the highest doses of the analog resulted in <10% of the control population; this was significantly lower than other evaluated tubulin-binding agents paclitaxel and pironetin (Fig. 3).

In summary, we synthesized α-functionalized pironetin analogs to evaluate structure-activity relationships of the C2 position since substitution at this position could potentially decrease the natural product's off-target covalent adduct formation. The analogs




**Scheme 1.** Retrosynthesis of α-functionalized pironetin analogs **2** (R = EWG group, PG = protecting group).