Bioorganic & Medicinal Chemistry Letters 23 (2013) 340–345

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Identification of inhibitors against interaction between pro-inflammatory sPLA2-IIA protein and integrin αvβ3

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ARTICLE INFO

Article history:
Received 10 August 2012
Revised 15 October 2012
Accepted 16 October 2012
Available online 24 October 2012

Keywords:
Inflammation
Inhibition of protein–protein interaction
sPLA2-IIA
Integrin

ABSTRACT

Increased concentrations of secreted phospholipase A2 type IIA (sPLA2-IIA), have been found in the synovial fluid of patients with rheumatoid arthritis. It has been shown that sPLA2-IIA specifically binds to integrin αvβ3, and initiates a signaling pathway that leads to cell proliferation and inflammation. Therefore, the interaction between integrin and sPLA2-IIA could be a potential therapeutic target for the treatment of proliferation or inflammation-related diseases. Two one-bead-one-compound peptide libraries were constructed and screened, and seven target hits were identified. Herein we report the identification, synthesis, and biological testing of two pyrazolylthiazole-tethered peptide hits and their analogs. Biological assays showed that these compounds were able to suppress the sPLA2-IIA–integrin interaction and sPLA2-IIA-induced migration of monocytic cells and that the blockade of the sPLA2-IIA–integrin binding was specific to sPLA2-IIA and not to the integrin.

Secreted phospholipase A2 group IIA (sPLA2-IIA) is an enzyme that hydrolyzes the sn-2 ester in the glyceroacyl phospholipids present in lipoproteins and cell membranes, resulting in release of arachidonic acid and lysophospho-lipids.[1] These products are subjected to further metabolism to form potent mediators which induce disease-related cellular processes, including inflammation, apoptosis, and atherogenesis.[2] As a result, sPLA2-IIA has been considered an important player in the inflammation-related diseases as well as a therapeutic target.[3] However, catalytically inactive sPLA2-IIA mutants are still able to enhance inflammatory processes via signal transduction involving kinases such as the extracellular responsive kinase 1 and 2 (ERK1/2), p38, and NF-κB.[4] Furthermore, potent inhibitors of the catalytic activity of sPLA2-IIA fail to demonstrate significant therapeutic effect for the treatment of inflammatory diseases such as rheumatoid arthritis[5] and asthma.[6] Thus, the paradox of the well known pro-inflammatory mechanism of the enzymatic function of sPLA2-IIA with the poor efficacy of its inhibitors in treating inflammatory diseases has prompted investigations into its other pathogenic roles.[7–10]

One plausible explanation for the paradox is that sPLA2-IIA may function as an 'inflammation-mediating' ligand by interacting with certain cell receptors through its non-catalytic site, causing retained pro-inflammatory signaling in cells. Indeed, there have been reports of interaction of sPLA2-IIA with cell receptors or cell surface substances to support the above hypothesis: for example, sPLA2-IIA binds to rodent M-type receptor[7] (human sPLA2-IIA does not bind to human M-type receptor)[8] and to heparan sulfate proteoglygans like glypican-1[9] and decorin in apoptotic human T-cells.[10] In addition, we recently discovered that sPLA2-IIA could interact with integrin αvβ3 and α4β1 and induce the pro-inflammatory activation of ERK1/2, as well as cell proliferation.[11] Therefore, based on our recent discovery of the signaling function of sPLA2-IIA, we postulate that interactions between sPLA2-IIA and integrins may serve as novel therapeutic targets for inflammation intervention and the treatment of related diseases, and that identification of the inhibitors of the interaction would be a practical stride towards employment of the new target. Hence, we initiated an inhibitor discovery effort in which we implemented peptide library synthesis, hit-identification via on-bead screening, and re-synthesis and validation of active compounds in biological assays. As a result, we identified small compounds that are able to inhibit the sPLA2-IIA–integrin αvβ3 interaction. These confirmed compounds and their inhibition activity could lead to a new strategy to treat diseases that stem from detrimental inflammation processes.

The One-Bead-One-Compound (OBOC) library is a powerful tool for identification of biologically active compounds or hit generation in drug discovery.[12,13a,14] The molecular diversity of the library can be easily increased simply by repetitive employment of the

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0960-894X/$ - see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.bmcl.2012.10.080

split-and-mix procedure and peptidyl chain elongation, and the resulting large molecular diversity would increase the possibility of active compounds present in the library. The identity of a compound on a bead can be conveniently established by Edman degradation[13b] or mass spectrometry protocol, depending upon the coding strategy utilized for the OBOC library. To further increase opportunities in binding to the target molecules as well as drug-likability of hit compounds, we capped the peptide chain with heteroatom-rich heterocyclic acids **1–10** (see Fig. 1 for structures of the capping agents). Two of them, **1** and **8** were synthesized in our laboratory.[15] The rest of these acids were obtained from commercial sources.

Two sets of OBOC libraries were prepared on 90 μm-diameter TentaGel beads according to a literature method (their general formula is shown in Fig. 2).[13a] Compounds in Set I consist of a tetrapeptide chain and compounds in Set II employ a hexapeptide one. Both libraries were synthesized on two-layered Tentagel beads with either L or D amino acids (see Supplementary data for isomer specification). The outer and inner layers on each bead contain almost the same peptide constitution except for the N-terminal residues: the terminal amino acid in the inner layer encodes for the heterocyclic capping moiety of the peptide chain on the outer layer. Considering that nineteen amino acids (D or L, cysteine not included) and ten different heterocyclic carboxylic acids were used to construct the libraries, the molecular diversity of the tetrapeptide library can reach ~1.3 million (10×19^4). For the hexapeptide library, the molecular diversity can reach ~15 million, as limited by the number of beads in the 5 g of Tentagel resin used in the library construction.

The synthesis of each library is executed in three phases as shown in Scheme 1. In phase I, the peptide chain is elongated from the free amine on bead (via **11** and **12**) to the desired length by repetition of split-coupling-mix operational cycles. In phase II, topological segregation of the two layers was achieved to give **14** by swelling of **13** in water followed by partial protection of the outer layer with an amount of FmocOSu inadequate for complete protection in the mixed solvent (DCM/Et_2O). Following layer formation, the inner layer of **14** was selectively Boc-protected giving **15**. Finally in phase III, the peptide chain in the outer layer was deprotected

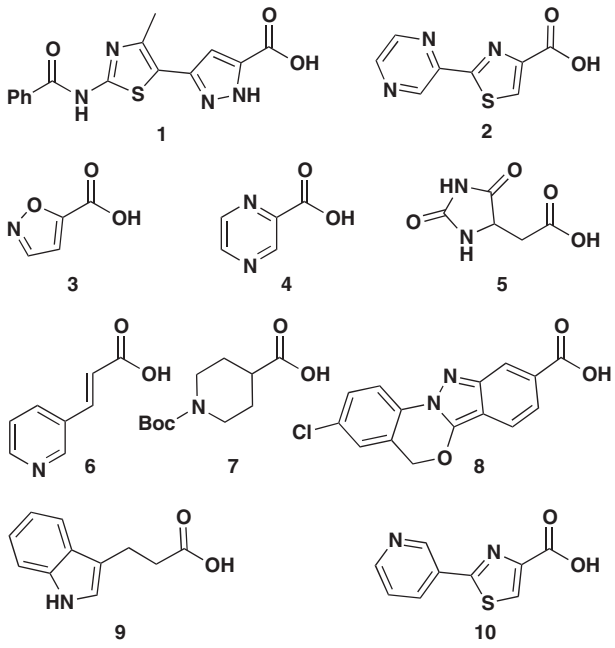

Figure 1. Capping agents employed in the target library.

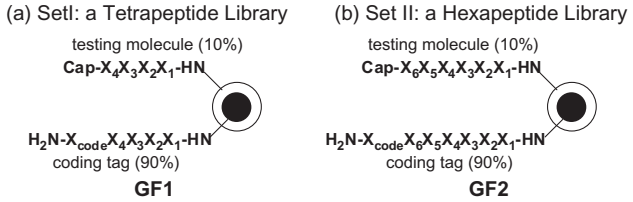

Figure 2. General formulas (GF) of the two sets of libraries used for on-bead screening (X = amino acid residue, D-isomers of R, N, L, F and D were used. Cap = heterocyclic carboxylic acid, Xcode = amino acid coded for a specific capping heterocycle).

and capped with the heterocyclic acids shown in Fig. 1 respectively in ten separate tubes. The hexapeptide OBOC library was synthesized in the same way except two more split-coupling-mix cycles were employed to gain the hexapeptide chain in the phase I stage.

With these encoded OBOC libraries in hand, a differential assay was conducted to identify hits that only bind to wild-type sPLA2-IIA, ideally only at its integrin-binding site. This assay consists of sequential incubations with an integrin-binding defective mutant sPLA2-IIA (R74E/R100E) and wild-type sPLA2-IIA, and image subtraction [12] to selectively visualize the beads that only contain the desired hits. The mutant sPLA2-IIA (R74E/R100E) was previously demonstrated to be unable to bind to integrin αvβ3. Our screening process involves a lengthy procedure, the principles of which are based on our already published strategies[12] and briefly illustrated in Scheme 2. In the petri dish shown in Scheme 2, all beads can be categorized into four types (A, B, C or D) according to the binding selectivity of the peptide on each bead. After incubating with biotinylated mutant sPLA2-IIA, there are two binding possibilities: some beads bind this protein and some do not. In this example, beads C and D bind the mutant protein and are stained blue by sequential treatment with antibiotin-alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), as shown in image 1 of Scheme 2. Image 1 was then recorded using a high-resolution scanner. Beads A and B do not bind mutant sPLA2-IIA. Because of that, beads A and B cannot be stained blue due to lack of the biotinylated mutant protein and therefore do not show up as blue beads in image 1. All the immobilized beads are then washed with PBS buffer solution to clear out the mutant protein free in solution and further incubated with wild-type sPLA2-IIA, resulting in three possibilities: some beads bind wild-type sPLA2-IIA only, like beads B; some bind both the mutant and wild-type protein, like beads D; and some do bind not either, like beads A. Since the mutant protein has only two amino acid mutations, much of its surface area might be similar to that of the wild-type sPLA2-IIA, including the catalytic site. The majority of the stained beads in image 2 can be categorized as beads D. However, our interest lies only in peptides that can recognize the uniqueness of the wild type sPLA2-IIA, the integrin-binding site by fitting into it. Since the mutant has lost its integrin-binding site, beads B, which do not bind the mutant and only bind the wild-type, are most likely to contain peptides that can recognize the integrin-binding site. After visualization and scanning, image 2 was obtained showing the above binding possibilities. By subtracting immobilized image 1 from immobilized image 2, only beads that bind with wild-type sPLA2-IIA remain visible in the differential image 3. Guided by image 3 in Scheme 2, immobilized beads B were selectively picked out of the petri dish and submitted for decoding of the peptide chain using an automated Edman degradation sequencer.

Although >100 beads showed up in the differential image (image 3), only twenty-four beads were selected for a quick decoding process. Upon Edman degradation some of the beads did not release residues above the instrument detection limit, but seven gave