Bioorganic & Medicinal Chemistry 21 (2013) 7981–7987

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Inhibitors of tissue-nonspecific alkaline phosphatase: Design, synthesis, kinetics, biomineralization and cellular tests

Julien Debray [a,b,†], Lei Chang [a,†], Stéphanie Marquès [a], Stéphane Pellet-Rostaing [a,c], Le Duy Do [b,d], Saida Mebarek [b], René Buchet [b,*], David Magne [b], Florence Popowycz [e,*], Marc Lemaire [a,*]

[a] Institut de Chimie et Biochimie Moléculaires et Supramoléculaires, UMR-CNRS 5246, Equipe Catalyse Synthèse Environnement, Université de Lyon, Université Lyon 1, F-69622 Villeurbanne, France
[b] Institut de Chimie et Biochimie Moléculaires et Supramoléculaires, UMR-CNRS 5246, Equipe Organisation et Dynamique des Membranes Biologiques, Université de Lyon, Université Lyon 1, F-69622 Villeurbanne, France
[c] Institut de Chimie Séparative de Marcoule, UMR-CNRS 5257, site de Marcoule, Université Montpellier 2, F-30207 Bagnols sur Cèze, France
[d] Department of Biochemistry, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 02-093 Warsaw, Poland
[e] Institut de Chimie et Biochimie Moléculaires et Supramoléculaires, UMR-CNRS 5246, Equipe Chimie Organique et Bioorganique, INSA Lyon, F-69621 Villeurbanne, France

ARTICLE INFO

Article history:
Received 31 July 2013
Revised 16 September 2013
Accepted 20 September 2013
Available online 17 October 2013

Keywords:
Alkaline phosphatase
Benzo[*b*]thiophen
Enzyme
Inhibitor
Levamisole
Vascular calcification

ABSTRACT

Chronic kidney disease (CKD) is associated with numerous metabolic and endocrine disturbances, including abnormalities of calcium and phosphate metabolism and an inflammatory syndrome. The latter occurs early in the course of CKD and contributes to the development and progression of vascular calcification. A few therapeutic strategies are today contemplated to target vascular calcification in patients with CKD: vitamin K2, calcimimetics and phosphate binders. However, none has provided complete prevention of vascular calcification and there is an urgent need for alternate efficient treatments. Recent findings indicate that tissue-nonspecific alkaline phosphatase (TNAP) may represent a very promising drug target due to its participation in mineralization by vascular smooth muscle cells. We report the synthesis of four levamisole derivatives having better inhibition property on TNAP than levamisole. Their IC_{50}, K_i and water solubility have been determined. We found that the four inhibitors bind to TNAP in an uncompetitive manner and are selective to TNAP. Indeed, they do not inhibit intestinal and placental alkaline phosphatases. Survival MTT tests on human MG-63 and Saos-2 osteoblast-like cells have been performed in the presence of inhibitors. All the inhibitors are not toxic at concentrations that block TNAP activity. Moreover, they are able to significantly reduce mineralization in MG63 and Saos-2 osteoblast-like cells, indicating that they are promising molecules to prevent vascular calcification.

1. Introduction

End-stage of renal disease [ESRD or stage-5 chronic kidney disease (CKD)] is associated with numerous metabolic and endocrine disturbances, including abnormalities of calcium and phosphate metabolism and an inflammatory syndrome. CKD contributes to the development and progression of vascular calcification. The prevalence of CKD has reached epidemic proportions with 10–13% of the population in Japan or Canada for instance.[1]

The coronary calcium score is recognized as a strong predictor of incident coronary heart disease in the general population[2] and even more in patients with ESRD[3–6] who develop exacerbated vascular calcification.[7] A few therapeutic strategies are today contemplated to target vascular calcification in patients with CKD: vitamin K2, calcimimetics and phosphate binders.[1] However, none has provided complete prevention of vascular calcification and there is an urgent need for alternate efficient treatments. A few years ago, it was still commonly thought that vascular calcification in ESRD was solely due to an increased Ca × Pi product resulting from hyperphosphatemia. However, several reports have challenged this point of view. For instance, medial calcification also occurs in aging and diabetes, when calcium and phosphate concentrations are in the normal range. Moreover, the mere increase in Ca × Pi product in vivo is not sufficient to induce any calcification in mice.[8] Medial calcifications much more likely result from the tissue-nonspecific alkaline phosphatase (TNAP) induced removal of inorganic pyrophosphate (PPi), which is a potent mineralization inhibitor. PPi is normally produced in the vasculature and other tissues to inhibit hydroxyapatite (HA) crystal formation. In bone and growth plate cartilage, PPi is physiologically removed by TNAP, an ectoenzyme expressed by osteoblasts and growth plate chondrocytes to promote mineralization. In humans, TNAP functional deficiency leads to severe hypomineralization and death in utero.[9] On the opposite, PPi deficiency due to mutations in the gene encoding the

* Corresponding authors. Tel.: +31 4 72 43 13 20; fax: 31 4 72 43 15 43.
E-mail address: rbuchet@univ-lyon1.fr (R. Buchet).
† Both Julien Debray and Lei Chang contributed equally.

0968-0896/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2013.09.053

PPi-producing enzyme NPP1 leads to severe, fatal arterial calcification.[10] Plasma PPi levels are negatively associated with vascular calcification in ESRD[11] which highlights the central role of TNAP. TNAP activity is significantly higher in vascular smooth muscle cells (VSMCs) from aortas of uremic rats as compared with control ones.[12] In this context, administration of PPi and/or inhibition of TNAP appear as particularly appealing strategies. PPi administration in uremic rodents strongly decreases vascular calcification.[11,13] However, PPi is a short-lived molecule in the circulation, with a half-life of about 30 min. It is therefore uncertain that PPi may be a suitable long-term treatment option in humans.[14] Therefore, we have privileged the strategy of TNAP inhibition.[15–18] The most potent commercial TNAP inhibitor known so far, levamisole, cannot be used in vivo, since 1 mM is necessary to block calcification in cultures of aortas from uremic rats, a level which cannot be achieved in vivo.[12] To our knowledge, only one laboratory in the United States developed new TNAP inhibitors having higher potency than levamisole and outstanding selectivity.[19–24] Recently, we patented a family of compounds showing a several-fold higher potency than levamisole to inhibit TNAP.[18] Their chemical structures[19–24] are completely different from that of our molecules. In this article, we report the synthesis of four levamisole derivatives having better TNAP inhibition properties as compared with levamisole. Their IC_{50}, K_i and water solubility have been determined. We found that the four inhibitors bind selectively to TNAP in an uncompetitive manner. They do not inhibit intestinal alkaline phosphatase (IAP) or placental alkaline phosphatase (PLAP). All inhibitors are not toxic at 10 μM concentration and are able to reduce the ascorbic acid (AA) and β-glycerophosphate (β-GP) induced mineralization in MG63 and in Saos-2 cells indicating that the development of TNAP inhibitors may be a promising strategy to prevent vascular calcification.

2. Results

2.1. Organic synthesis

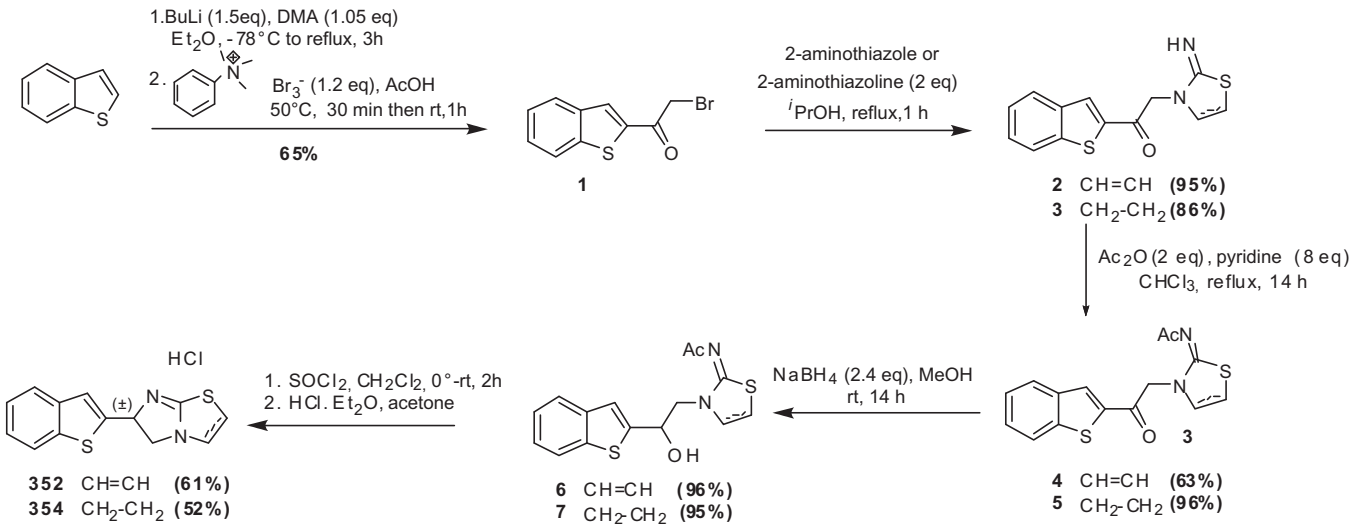
In 1977, a series of *meta*- and *para*-substituted phenyl derivatives of (±)-2,3-dehydrotetramisole and (±)-tetramisole was synthesized.[25,26] Following the synthetic methodology already described above, benzo[*b*]thiopheno-2,3-dehydrotetramisole **352** and (±)-tetramisole **354** were obtained in five steps from the known 3-(2-bromoacetyl)benzo[*b*]thiophene **1** previously synthesized from benzo[*b*]thiophene as reported in the literature (Scheme 1).[27,28]

352 was prepared in an overall yield of 22% by condensation of **1** with the 2-aminothiazole followed by the acetylation of **2**, sodium borohydride reduction of **4** and ring closure of **6** with thionyl chloride. A similar sequence of reactions was employed for the condensation of the bromomethyl aryl ketone **1** with 2-aminothiazoline. Following the similar synthetic sequence developed for **352**, the intermediate **3** was converted into levamisole derivative **354**. To increase the hydrophilic character of the **352** benchmark compound, amino group was introduced from the first step of the synthetic sequence starting from 2-acetyl-3-aminobenzo[*b*]thiophene.[29] To complete the series of compounds **352-**NO_2 and **352-**NH_2 were prepared (Fig. 1) (Synthetic scheme and analytical data are available in the Supplementary data).

2.2. Inhibition and solubility properties of benzothiophenyl derivatives as compared to those of levamisole

The inhibition property of each compound was determined by measuring TNAP activity at alkaline pH (optimum conditions) and at physiological pH, IC_{50} values determined at both pH (Table 1) indicated that all four synthetized inhibitors (**352**, **354**, **352-**NO_2 and **352-**NH_2) have better inhibition properties than levamisole. Indeed their IC_{50} values either at pH 10.4 or at pH 7.8 were significantly lower as compared with those of levamisole. Standard internal reference levamisole has been also included consistently all along the biological assays. Each compound behaves in an uncompetitive manner and is selective to TNAP. At 400 mM—that is, thirteen to hundred times more to inhibit TNAP- all compounds did not inhibit IAP or PLAP indicating specificity of the inhibition (Table 1).

Assuming that each inhibitor acts in an uncompetitive manner having Michaelis–Menten kinetics, K_i amounted to 1.3 ± 0.1 μM for **352-**NO_2—the best inhibitor-as compared with that of levamisole which was 30.5 ± 0.5 μM while other have K_i ranging from 3.9 ± 0.2 μM (**352**), 5.0 ± 0.2 μM (**354**) to 10.3 ± 0.1 μM (**352-**NH_2), confirming that all synthesized inhibitors can inhibit TNAP at thirty (**352-**NO_2) to three time (**352-**NH_2) lower concentration than levamisole (Table 1). Solubility property is an important factor which can control the accessibility of the inhibitor in hydrophobic or hydrophilic binding sites. Therefore we synthesized a series of inhibitors having different water solubility. The



Scheme 1. Reaction scheme.