European Journal of Medicinal Chemistry 102 (2015) 403–412

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Synthesis of new indole-based bisphosphonates and evaluation of their chelating ability in PE/CA-PJ15 cells

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ARTICLE INFO

Article history:
Received 1 April 2015
Received in revised form
22 July 2015
Accepted 8 August 2015
Available online 12 August 2015

Keywords:
Indole based bisphosphonate
Cytosolic calcium
Ene reaction
Chelating ability assessment

ABSTRACT

Bisphosphonates are the most important class of antiresorptive agents used against osteoclast-mediated bone loss, and, more recently, in oncology. These compounds have high affinity for calcium ions (Ca^{2+}) and therefore target bone mineral, where they appear to be internalized selectively by bone-resorbing osteoclasts and inhibit osteoclast function. They are extensively used in healthcare, however they are affected by severe side effects; pharmacological properties of bisphosphonates depend on their molecular structure, which is frequently the cause of poor intestinal adsorption and low distribution. In this work we synthesized six novel bisphosphonate compounds having a variably substituted indole moiety to evaluate their extra- and intracellular calcium chelating ability in PE/CA-PJ15 cells. Preliminary *in silico* and *in vitro* ADME studies were also performed and the results suggested that the indole moiety plays an important role in cell permeability and metabolism properties.

1. Introduction

Bisphosphonates (BPs) are analogs of naturally occurring pyrophosphates and represent an important class of drugs, used to treat conditions such as osteoporosis, Paget's syndrome and some tumors [1,2]. Similarly to inorganic pyrophosphate, BPs antagonize the dissolution of hydroxyapatite crystals and the metastatic calcification of tissues, however unlike their endogenous analogs they are not cleaved by pyrophosphatase [3,4]. The biological activity of BPs largely depends on the Ca^{2+}-chelating properties of the two phosphate groups. As it is widely known, calcium is used in cellular signaling: in eukaryotic cells the ion is sequestered in cellular organelles (such as endoplasmic reticulum and mitochondria) and it is released according to the cell activation [5]. Cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_c$ is very low ($\approx$100 nM) compared to that in the extracellular medium ($\approx$1 mM). The maintenance of such a calcium concentration gradient between the cytosol and other compartments is crucial for cells survival [6]. Calcium homeostasis is finely tuned through various mechanisms [7]; an uncontrolled Ca^{2+} signal may alter secretion, contraction, protein activity, apoptosis, proliferation and other critical processes [8].

BPs can be distinguished in two classes: the non-nitrogen-containing bisphosphonates (NNBPs) and the nitrogen-containing bisphosphonates (NBPs) [9]. Etidronate (ETD) and clodronate (CLD) (Fig. 1) belong to the NNBPs family, and they are known to block ATP-dependent enzymes able to induce the death of the osteoclast [10]. Alendronate (ALN), risedronate (RSD), and pamidronate (PMD) (Fig. 1) belong to the NBPs class, and are inhibitors of the farnesyl pyrophosphate synthase [2]. These drugs inhibit the prenylation of GTPase-dependent proteins (Ras, Rab, Rho and Rac) that are essential for the osteoclast's survival. The deterioration of cytoskeleton of these cells does not allow the formation of the ruffled border, causing their apoptosis [11,12].

Survival time, proliferation, adhesion and migration are the main effects of NBPs on tumor cell lines "*in vitro*" [13,14] and most of these effects are due to the inhibition of farnesyl pyrophosphate synthetase [15]. In addition, the inhibition of protein prenylation may alter the viability and the proliferation of endothelial cells *in vitro* [16]. Studies on mice indicate that NBPs inhibit the formation of bone metastases and decrease bone mass *in vivo* [16,17], suggesting a relationship between their antitumor effects and their cellular uptake *in vivo*. NBPs induce a pre-apoptotic action on

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http://dx.doi.org/10.1016/j.ejmech.2015.08.019
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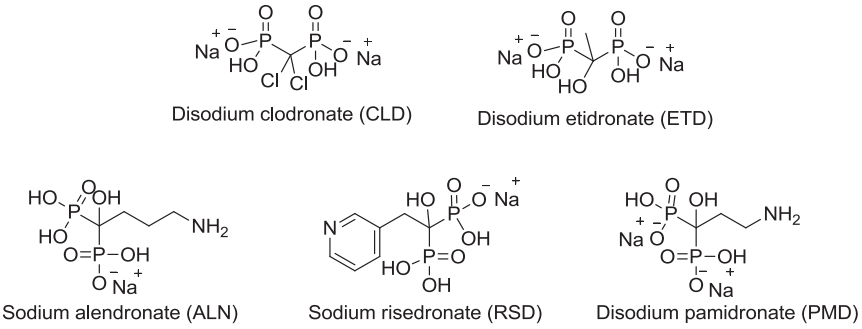

Fig. 1. Chemical structure of commercially available bisphosphonate drugs in their sodium salt form (clodronate, etidronate, alendronate, risedronate, and pamidronate).

neoplastic cancer cells and inhibit the mechanisms regulating the cellular proliferation and adhesion [14,15].

The major drawback of NBP drugs is their high hydrophilicity and polarity. Thus, many common NBP drugs demonstrate poor cell-membrane permeability towards tumor cells and other non-endocytic cells [18,19]. The uptake and binding to bone mineral is determined by the presence of the bisphosphonates moiety, while the substituents bound to the P–C–P moiety are thought to be essential to ensure satisfactory antiresorptive potency. Thus, small changes in the substituents of the P–C–P moiety may influence the resorption properties [20].

Another consequence of the high hydrophilicity of NBPs is their metabolic stability. Indeed, they are not metabolized by CYP450 or other phase I enzymes [21], and are mostly eliminated unchanged in urine [20]. On one hand, an extensive metabolism of a drug might jeopardize its pharmacological activity; on the other hand, when drugs are substrates for CYP450 enzymes, their half-life, as well as the pharmacological and toxicity effects, can be modulated by synthetic modifications.

As mentioned above, residual bisphosphonates which are not taken up by the bones are excreted in urine without being unmetabolized. As a consequence, a common adverse event related to bisphosphonate drugs is nephrotoxicity. In fact, pharmacokinetic and pharmacodynamic properties (see Table 1 for pK_a, LogP, and oral bioavailability data), modulate the bisphosphonates accumulation which is related to renal histopathology. Even though a low dose is usually well tolerated, the high dose required in an oncology setting is often correlated to nephrotoxicity [22]. In particular, the major toxicity effects seem correlated to intravenous administration of BPs. In addition, orally administered BPs are often correlated with adverse effects on gastrointestinal tract, such as ulceration in esophagus, stomach and duodenum. Other adverse events such as acute-phase reactions, and the osteonecrosis of the jaw have also been observed [23]. Thus, the improvement of the pharmacokinetic properties of bisphosphonates retaining the desired biological effect represents a valuable strategy to obtain novel series of safer bisphosphonate drugs.

Thus, in this work six novel indole-based bisphosphonates (IBPs) (compounds **1a**–**f**, Fig. 2) were synthesized and investigated for their ability in chelating extracellular and cytosolic calcium in the PE/CA P-J15 cells as a function of both the nature and the position of the substituent in the indole nucleus. In particular, owing to the interesting role played by fluorine in bioactive molecules [29–31], four fluorinated compounds were investigated. The PE/CA-PJ15 cells are obtained from human oral squamous cancer cells; they are similar to epithelial cells and express laminin and cytokeratins [32] and are responsible for about 90% of head and neck tumors. Our results showed that two out of six indole-bisphosphonates were significantly more effective than the commercially available ALN and CLD in chelating Ca^{2+}. The *in silico* evaluation of the passive cell membrane permeation ability suggested that the Ca^{2+}-binding efficacy of these compounds may be related to an increased cell permeability. Finally, the metabolic stability in human liver microsomes was determined for the most promising compounds, which, conversely to the ALN and CLD compounds, were rapidly metabolized.

Table 1
Experimental pK_a, LogP and oral bioavailability data of commercial BPs.

Comp.	pK_{a1-5}[a]	LogP[b] [24]	Oral bioavailability %[b] [25]
CLD	1.7, 2.1, 5.7, 8.3 [25]	−2.4	1–2
ETD	1.7, 2.5, 7.2, 10.8 [26]	−3.8	3–7
ALN	1.3, 2.2, 6.4, 11.0, 11.8 [27]	−4.3	0.75
RSD	1.6, 2.2, 5.9, 7.1, 11.7 [28]	−3.6	0.65
PMD	1.2, 1.9, 6.0, 10.2, 12.1 [27]	−4.7	0.3

[a] Related to respective acid form.
[b] Referred to commercial salt form.

2. Results and discussion

2.1. Synthesis of indole-based bisphosphonates

The indole nucleus is a well-established pharmacophore present in many synthetic and natural drugs with remarkable biological activities [10] and indole containing bisphosphonates have already been assessed for their anti-bone resorptive activity in bone marrow osteoclast culture [33].

As previously reported, 3-methyleneindolines are valuable building blocks for 3-functionalized indole derivatives due to the remarkable reactivity of the exocyclic carbon–carbon double bond through the “ene” reaction [34,35]. These findings led us to consider their use for the synthesis of new regioselectively substituted tetraethyl [3-(1*H*-indol-3-yl)propane-1,1-diyl] bis(phosphonate) derivatives **1a**–**f** with Ca^{2+} chelating ability and improved cell permeability (Fig. 2). The *N*-Boc protected 3-methyleneindolines **5a**–**f** were prepared in four steps, starting from commercially available substituted anilines (Scheme 1). When not commercially available, *o*-bromoanilides **3** were obtained by metalation of the *N*-Boc-protected anilines **2**, followed by treatment of the resulting dilithiated anilide intermediate with CBr_4. NaH-promoted deprotonation of anilide **3** in DMF, followed by the reaction with propargyl bromide, gave the corresponding *N*-propargyl derivatives **4** in good yield. Finally, the reductive radical cyclization of *N*-bromoaryl-*N*-propargylcarbamate with tributhyltin hydride in refluxing benzene, in the presence of AIBN initiator, provided the expected 3-methyleneindoline **5** in satisfactory yields. 3-Methyleneindolines **5** are relatively stable at room temperature and can be purified by standard chromatographic