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Bone 37 (2005) 349 – 358

BONE

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# Phosphonocarboxylate inhibitors of Rab geranylgeranyl transferase disrupt the prenylation and membrane localization of Rab proteins in osteoclasts in vitro and in vivo

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Received 6 December 2004; revised 4 March 2005; accepted 22 April 2005
Available online 11 July 2005

**Abstract**

Nitrogen-containing bisphosphonate drugs such as risedronate act by inhibiting farnesyl diphosphate synthase, thereby disrupting protein prenylation in osteoclasts. We recently found that an anti-resorptive phosphonocarboxylate analogue of risedronate, 3-PEHPC (previously referred to as NE10790), selectively prevents prenylation of Rab GTPases in vitro by specifically inhibiting Rab geranylgeranyl transferase. In this study, we demonstrate that unprenylated Rab6 could be detected in J774 cells after treatment with 3-PEHPC or risedronate for as little as 4 h, and reached 50% after 24 h. Furthermore, treatment of J774 cells or osteoclasts with either 3-PEHPC or risedronate disrupted membrane association of several Rab family proteins. Like risedronate, the effects of 3-PEHPC are likely to be restricted to osteoclasts in vivo, since both risedronate and 3-PEHPC inhibited Rab prenylation in osteoclasts, but not in general bone marrow cells, when administered to rabbits in vivo. Analysis of two new phosphonocarboxylate analogues of 3-PEHPC (3-PEPC and 2-PEPC) revealed that, first, the geminal hydroxyl group is not essential for inhibition of Rab prenylation by phosphonocarboxylates, but does contribute to their anti-resorptive potency, most likely by enhancing their affinity for bone mineral. Second, the position of the nitrogen in the side chain of phosphonocarboxylates is crucial for their ability to inhibit Rab prenylation and hence to inhibit bone resorption. In addition, there is a good correlation between the ability of the phosphonocarboxylates to inhibit Rab prenylation and to inhibit bone resorption in vitro, indicating that these compounds are a new class of pharmacological agents that inhibit bone resorption by specifically preventing prenylation of Rab proteins. Furthermore, although phosphonocarboxylates are analogues of bisphosphonates, the structure–activity relationships of phosphonocarboxylates for inhibiting Rab geranylgeranyltransferase appear to differ from the structure–activity relationships of bisphosphonates for inhibiting farnesyl diphosphate synthase.


*Keywords:* Bisphosphonate; Phosphonocarboxylate; Osteoclast; Protein prenylation; Rab GGTase

## Introduction

Protein prenylation is a post-translational modification involving the transfer of either a farnesyl or geranylgeranyl isoprenoid lipid to the c-termini of specific target proteins, chiefly small GTPases of the Ras, Rho, and Rab families. This modification is critical for the function of these proteins, since it is required for membrane association and for specific protein–protein interactions [1]. The process of prenylation is carried out by one of three distinct protein:-prenyl transferase enzymes, the specificity being determined by the prenylation motif in the protein substrate. These

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8756-3282/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bone.2005.04.021

enzymes are protein:farnesyl transferase (FTase), which farnesylates proteins such as Ras and lamins; protein:geranylgeranyl transferase I (GGTase I), which geranylgeranylates mainly small GTPase proteins of the Rho family (e.g., Rho, Rac, and Cdc42); and Rab protein:geranylgeranyl transferase (Rab GGTase), which specifically geranylgeranylates small GTPases of the Rab family [2] that are involved in the trafficking of intracellular vesicles [3]. We recently identified 3-PEHPC [2-(3-pyridinyl)-1-hydroxyethylidene-1,1-phosphonocarboxylic acid; previously referred to as NE10790], a phosphonocarboxylate (PC) compound (Fig. 1), as the first specific inhibitor of Rab GGTase. 3-PEHPC prevents the prenylation of Rab proteins in vitro, without affecting prenylation of Ras or Rho family proteins [4]. However, the effect on the membrane association of Rab proteins following inhibition of Rab prenylation by 3-PEHPC or RIS has not been studied.

3-PEHPC is an analogue of the bisphosphonate drug risedronate (RIS), in which one of the phosphonate groups is replaced by a carboxylate group. Bisphosphonates (BPs), in particular those containing nitrogen (N-BPs), are the most widely used and effective treatments for diseases of bone metabolism characterized by excessive bone resorption, including post-menopausal osteoporosis, Paget's disease and tumor-associated osteolysis [5]. These drugs act by directly and selectively inhibiting the activity of osteoclasts, the cells responsible for bone resorption. Following in vivo administration, BPs rapidly target bone mineral due to their P-C-P backbone structure, which provides these drugs with a high affinity for calcium ions (reviewed by Rogers et al. [6]). During bone resorption, BPs are liberated from the bone surface and internalized by osteoclasts [7,8]. N-BPs disrupt osteoclast function by inhibiting farnesyl diphosphate (FPP) synthase, blocking the synthesis of both farnesyl and geranylgeranyl isoprenoids and thereby preventing the prenylation of small GTPases required for osteoclast function [9,10]. By inhibiting FPP synthase, N-BPs block prenylation of all small GTPases, although there is evidence that the effects of N-BPs on osteoclasts are mainly due to loss of geranylgeranylation of Rho family GTPases that are prenylated by GGTase I [11–13].

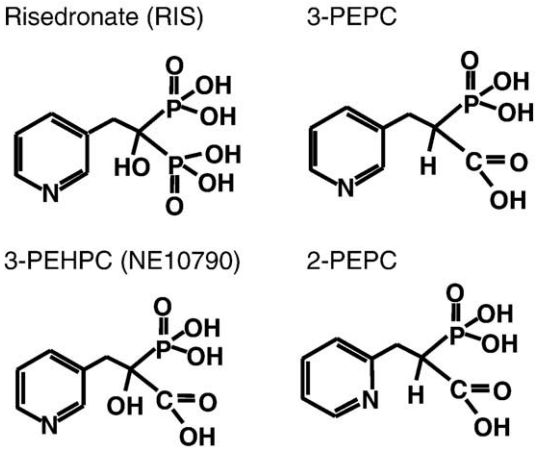


Fig. 1. Structure of compounds. Structure of the bisphosphonate risedronate [RIS; 2-(3-pyridinyl)-1-hydroxyethylidene-1,1-bisphosphonic acid] and the phosphonocarboxylate analogues 3-PEHPC [2-(3-pyridinyl)-1-hydroxyethylidene-1,1-phosphonocarboxylic acid], 3-PEPC [2-(3-pyridinyl)-1-ethylidene-1,1-phosphonocarboxylic acid], and 2-PEPC [2-(2-pyridinyl)-1-ethylidene-1,1-phosphonocarboxylic acid].

Like N-BPs, 3-PEHPC also inhibits bone resorption in vitro and in vivo, [4,14,15], indicating that, in addition to Rho proteins, prenylated Rabs are also crucial for osteoclast function [4]. However, 3-PEHPC is a much weaker inhibitor of bone resorption than RIS, probably due in part to the reduced affinity of 3-PEHPC for bone compared to RIS, as a result of the replacement of one of the phosphonate groups with a carboxylate group [15]. Currently, little is known about the relationship between molecular structure and inhibition of Rab GGTase by 3-PEHPC or other PCs [4]. By contrast, structure–activity studies using BPs in several different model systems has yielded valuable information regarding the structural features of BPs essential for their activity and potency for inhibiting FPP synthase. For example, as well as being important for binding to bone, the presence of two phosphonate groups is required for interaction with the molecular target [14–16], emphasized by the inability of 3-PEHPC to inhibit FPP synthase [4]. By contrast, a geminal hydroxyl group in nitrogen-containing BPs increases bone affinity [17], but does not appear to influence the ability of BPs to act at the cellular level [18]. Of most importance regarding potency, however, is the structure of the $R^2$ side chain. Potent N-BPs are all characterized by the presence of a nitrogen in either an alkyl (e.g., alendronate) or heterocyclic (e.g., RIS) side chain. The 3-dimensional orientation of this basic nitrogen moiety is critical for effective inhibition of FPP synthase [10], since minor modifications to the side chain that alter the position of the nitrogen in relation to the P-C-P backbone can dramatically affect potency [10,16,19].

In this study, we examined the effect of RIS and 3-PEHPC on Rab prenylation in vitro and in vivo, and on the association of Rabs and other small GTPases with specific subcellular membranes. Since 3-PEHPC could represent a new class of pharmacological agents for inhibiting Rab prenylation, we also examined the role of molecular structure in inhibition of Rab GGTase and inhibition of bone resorption by PCs.

## Materials and methods

### *Reagents*

RIS, 3-PEHPC, 3-PEPC [2-(3-pyridinyl)-1-ethylidene-1,1-phosphonocarboxylic acid], and 2-PEPC [2-(2-pyridinyl)-1-ethylidene-1,1-phosphonocarboxylic acid; Fig. 1] were from Procter & Gamble Pharmaceuticals, Cincinnati,