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N-Oxide derivatives of 3-(3-pyridyl)-2-phosphonopropanoic acids as potential inhibitors of Rab geranylgeranylation

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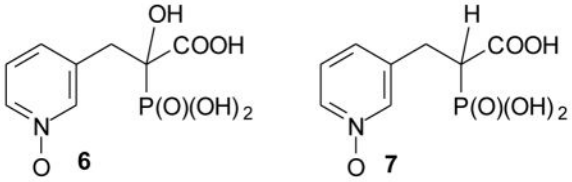
Abstract

The *N*-oxide derivatives of [2-(3-pyridinyl)-1-hydroxyethylidene-1,1-phosphonocarboxylic acid (or PEHPC) and [2-(3-pyridinyl)-1-ethylidene-1,1-phosphonocarboxylic acid (or PEPC) have been prepared and evaluated for their activity against several enzymes which utilize isoprenoids. The parent pyridines are known inhibitors of GGTase II, but the *N*-oxide derivatives show no improvement in biological activity in assays with the isolated enzyme. However, the PEHPC *N*-oxide did induce significant accumulation of intracellular light chain in myeloma cells, consistent with inhibition of Rab geranylgeranylation.

Graphical Abstract



Keywords

pyridyl *N*-oxides; GGTase II; Rab GGTase; apoptosis; myeloma; bioassay

The Rab family of small GTPases plays key roles in mediating intracellular trafficking events. These proteins are geranylgeranylated by the enzyme geranylgeranyl transferase II

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Supplementary data

Supplementary data (representative experimental procedures, NMR spectra, and bioassay protocols) associated with this article can be found in the online version, at

(GGTase II) and mutant forms of Rabs that are unable to be geranylgeranylated are mislocalized and therefore nonfunctional.[1] We have hypothesized that agents which impair Rab geranylgeranylation, either directly via inhibition of GGTase II,[2] or indirectly via depletion of GGPP,[3,4] would result in disruption of monoclonal protein secretion in human myeloma cells. We have shown that disruption of Rab geranylgeranylation leads to an accumulation of monoclonal protein in the endoplasmic reticulum, induction of the unfolded protein response pathway, and apoptosis.[5,6] Because myeloma cells are so heavily engaged in secretion of antibodies, specific inhibitors of GGTase II may represent a novel therapeutic strategy for treatment of myeloma or other diseases characterized by excessive protein secretion.

Although several types of GGTase II inhibitors are known,[7–10] we were particularly intrigued by the phosphonocarboxylate family.[11] Both PEHPC (**1**, also known as NE-10790) and PEPC (**2**) can be viewed as analogues of the bisphosphonate risedronate (**3**).[12] However, while risedronate is a potent inhibitor of farnesyl diphosphate synthase (FDPS, IC_{50} ~ 6 nM)[11] and is in clinical use for the treatment of osteoporosis,[13] compounds **1** and **2** show little activity against FDPS but do inhibit selectively the downstream enzyme GGTase II. Reported IC_{50} values for PEHPC against GGTase II range from ~32 to ~600 uM. [14–16] Furthermore, formal deletion of the OH group (i.e. compound **2**) does not greatly diminish activity against GGTase II,[11] and the enantiomers of **1** differ in activity by only ~4-fold.[16] In contrast the phosphono carboxylate **4**, also known as IPEHPC and viewed as an analogue of the bisphosphonate minodronate (**5**),[12,17] shows significantly greater potency even as the racemate, and the (+)-enantiomer **4** is ~60-fold more active than the (−)-enantiomer.[15] However, the (+)-enantiomer also shows weak inhibition of at least one other enzyme involved in isoprenoid biosynthesis, probably geranylgeranyl diphosphate synthase (GGDPS).[16]

Studies of IPEHPC have revealed that it inhibits the second geranylgeranylation of Rab proteins,[16] which suggests that it might be feasible to inhibit processing of some Rab proteins selectively over others. Because both PEHPC and IPEHPC inhibit this second geranylgeranylation but not the first, they may bind in an enzyme complex that includes both GGTase II and a Rab escort protein (REP) in such a way that the reorganization of the complex necessary for the second transfer is prevented. It has been suggested that complexation occurs in a large carvern in the protein, but the specific structural features that favor this binding are not yet clear. In an effort to advance understanding of structure activity relationships, and ultimately to obtain more potent inhibitors of GGTase II, we have begun to prepare derivatives of PEHPC and PEPC. In this paper we report the preparation and biological activity of the *N*-oxide derivatives of these compounds. The *N*-oxide introduces an N-1 substituent while yielding analogues that preserve a lone pair near the placement of the lone pair in the original pyridyl nitrogen, and at the same time are still formally neutral with respect to the pyridyl ring. While other derivatives can be imagined that accomplish both of these goals, they would require significantly more deep-seated changes to the structure. It was our expectation that synthesis and biological evaluation of the *N*-oxides would encourage, or discourage, preparation of less accessible analogues (or

mimics) of these pyridine derivatives. Thus the target compounds became the *N*-oxides **6** and **7**.

Synthesis of two *N*-oxide analogues of PEHPC is shown in Figure 3. Pyridine aldehyde **8** was condensed with ethyl ester **9** to afford the known keto ester **10**,[18] which was further treated with diethyl phosphite to give the key intermediate **11**. Compound **11** was allowed to react with *m*CPBA to oxidize the pyridine ring and give *N*-oxide **12** in a reasonable yield. Acid hydrolysis of *N*-oxide **12** converted it to the corresponding acid **13**, but only in low yield. While some carboxylate phosphonate triesters can be converted to the corresponding triacids by prolonged treatment with base,[19] that approach was not feasible in this series. Instead, treatment of the triester **12** with base resulted in a smooth rearrangement to the corresponding phosphate **14**. Based on analysis of the ^{31}P NMR spectrum, acid catalyzed hydrolysis of phosphate triester **14** gave the corresponding phosphate monoester **15**, but this compound was not examined further.

The GGTase II inhibitor PEPC (**2**), which has a potency similar to PEHPC,[20] was pursued through a parallel synthesis. However, it was challenging to obtain the target molecule **2** following a literature procedure,[21] perhaps due to the poor solubility of the commercially available hydrochloride salt **16** in organic solvents. To overcome this issue, the hydrochloride salt was first neutralized by treatment with $NaHCO_3$ to give compound **16** as the free base which was then added to a solution of the anion of triethyl phosphonoacetate (**15**) *in situ*.[22] The reaction mixture was allowed to stir overnight to form the PEPC precursor **17** in reasonable yield. With the key intermediate **17** in hand, part of the material was converted directly to PEPC (**2**) by hydrolysis while the remaining material was treated with the *m*CPBA to generate *N*-oxide **18**. Hydrolysis of triester **18** by treatment with HCl gave the corresponding acid **7**. At this point, the known GGTase II inhibitors, PEHPC (**1**), and PEPC (**2**) and their new *N*-oxide analogues **6** and **7** were tested for their relative biological activity.

The *N*-oxides **6** and **7**, as well as the parent compounds **1** and **2**, were tested for their ability to inhibit FDPS or GGTase II in *in vitro* enzyme assays.[3] As shown in Table 1, these compounds do not potently inhibit either enzyme. Despite these compounds displaying similar activity against both enzymes, only evidence of GGTase II inhibition was observed in cell culture studies. Immunoblot studies were performed to assess the effects of these compounds on protein geranylgeranylation in RPMI-8226 human myeloma cells. Rap1a is a substrate of GGTase I and an antibody was used which detects only unmodified protein. Rab6 is a representative Rab protein and is therefore a substrate of GGTase II. For Rab6, a Triton X-114 lysis protocol was used to generate a detergent (membrane) fraction.[3] With disruption of Rab geranylgeranylation, there is a decrease in membrane-bound protein. Lovastatin, an HMG-CoA reductase inhibitor which globally disrupts protein prenylation, was used as a control. As shown in Figure 5A, none of the bisphosphonates induce an accumulation of unmodified Rap1a. As expected, PEHPC (1) induces a decrease in the amount of membrane-bound Rab6. The *N*-oxide **6** and PEPC (**2**) diminished the level of membrane-bound Rab6 to a lesser extent while the *N*-oxide **7** did not decrease membrane-bound Rab6 levels. As we have demonstrated previously, both lovastatin and PEHPC (**1**)

induce apoptosis (as indicated by PARP and caspase 3 cleavage) as well as ER stress (calnexin cleavage).[5,6] Interestingly, although compound **7** does not appear to alter significantly Rab6 levels in the membrane fraction, it does induce cleavage of PARP, caspase 3, and calnexin to a similar extent as the parent compound **2**, suggesting there may be off-target effects. Finally, the ability of these compounds to disrupt monoclonal protein trafficking (a functional read-out of impairment of Rab geranylgeranylation[3,5]) was examined. As shown in Figure 5B, PEPHC and its *N*-oxide derivative **6** induce an accumulation of intracellular light chain while PEPC and its *N*-oxide derivative **7** do not significantly alter light chain trafficking, which is consistent with the weaker ability of the latter two compounds to diminish Rab geranylgeranylation.

While it is somewhat disappointing that the new N-oxides are not more potent inhibitors of GGTase II in assays with the isolated enzyme, at the same time it is significant that the PEHPC *N*-oxide **6** does have cellular activity consistent with inhibition of Rab geranylgeranylation. This suggests that larger substituents at the pyridyl nitrogen might be tolerated or even afford greater potency. Studies along these lines are underway and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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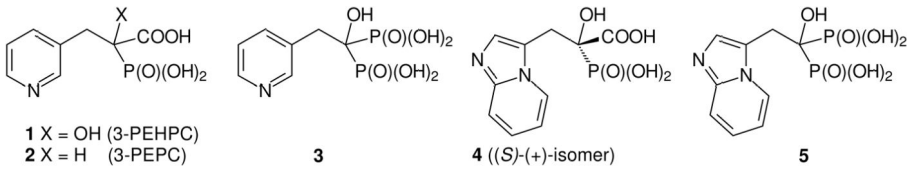

Figure 1. Pyridyl bisphosphonates and the corresponding carboxy phosphonates

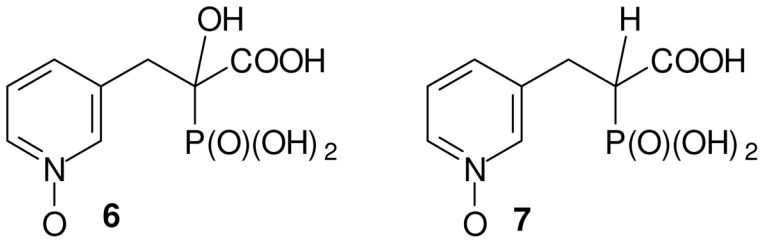

Figure 2. ***N*-Oxide derivatives of PEHPC (6) and PEPC (7)**

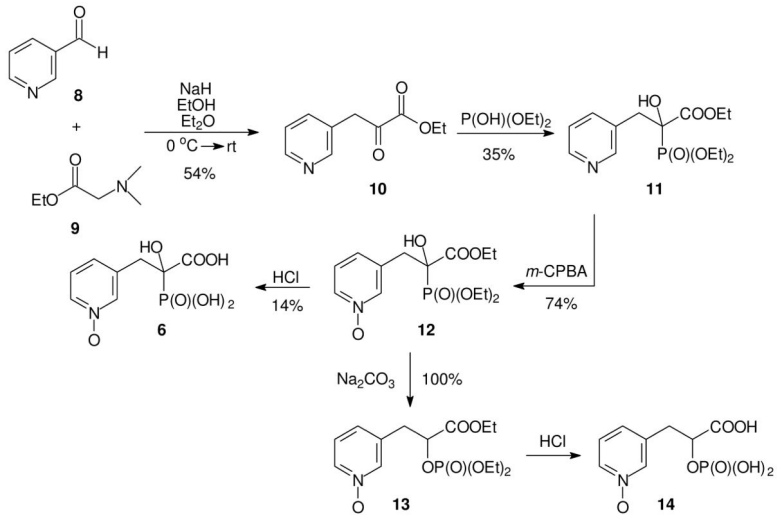

Figure 3. Synthesis of *N*-oxide analogues of PEHPC

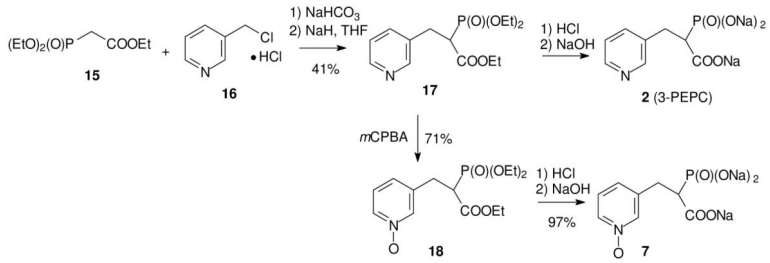

Figure 4. Synthesis of PEPC and its *N*-oxide analogue

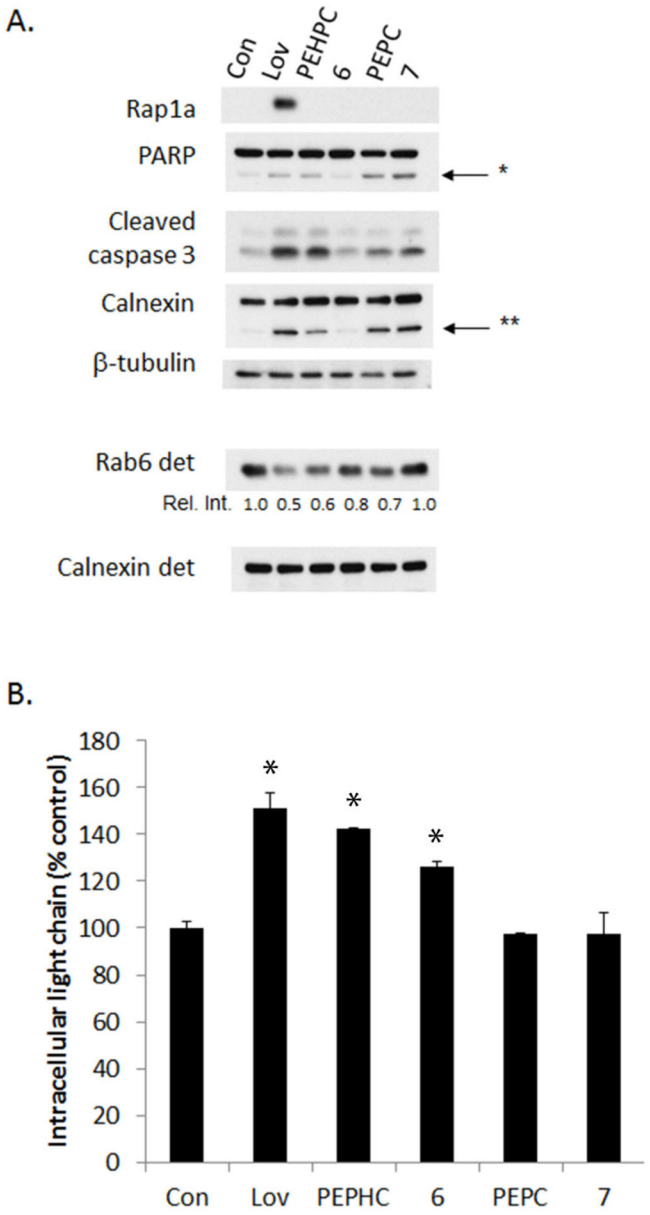

Figure 5. Effects of PEHPC derivatives in myeloma cells

RPMI-8226 cells were incubated for 48 hours in the presence or absence of lovastatin (20 μM, Lov), PEHPC (5 mM), PEPC (5 mM), or the *N*-oxides **6** and **7** (5 mM). A) Cells were lysed using RIPA buffer to generate whole cell lysate or with Triton X-114 to generate a detergent (membrane) fraction and immunoblot analysis was performed. The Rap1a antibody detects only unmodified protein. β-Tubulin was used as a loading control for whole cell lysate and calnexin was used as the loading control for the detergent fraction. * Denotes the PARP cleavage product while ** denotes the calnexin cleavage product. The gels are representative of two independent studies. B) Intracellular lambda light chain concentrations were determined via ELISA. Data are expressed as percentage of control (mean + SD, n=3).

The * denotes $p<0.05$ per unpaired two-tailed *t*-test and compares treated cells to untreated control cells.

Table 1

	FDPS IC_{50} (mM)	GGTase II IC_{50} (mM)
1 (PEHPC)	0.2	0.7
6	2	1.8
2 (PEPC)	1	1.1
7	>2	0.8