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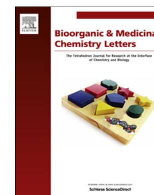

Phosphinic acid-based inhibitors of tubulin polyglutamylases

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ABSTRACT

Tubulin is subject to a reversible post-translational modification involving polyglutamylation and deglutamylation of glutamate residues in its C-terminal tail. This process plays key roles in regulating the function of microtubule associated proteins, neuronal development, and metastatic progression. This study describes the synthesis and testing of three phosphinic acid-based inhibitors that have been designed to inhibit both the glutamylating and deglutamylating enzymes. The compounds were tested against the polyglutamylase TTLL7 using tail peptides as substrates (100 μM) and the most potent inhibitor displayed an IC_{50} value of 150 μM. The incorporation of these compounds into tubulin C-terminal tail peptides may lead to more potent TTLL inhibitors.

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The cytoskeleton of the eukaryotic cell plays key roles in determining cellular organization, shape, motility, and life cycle and is therefore absolutely essential for cellular survival. The major structural components of the cytoskeleton are microtubules, which are hollow cylindrical polymers formed by the self-assembly of α- and β-tubulin monomers.[1] In the assembled microtubule, the C-termini, or tails, of the tubulin subunits are projected outward into solution and contribute to defining the surface of the filament. These tails are subject to reversible post-translational modifications (PTM's) such as detyrosination, polyglutamylation, and polyglycylation.[2,3] It is becoming increasingly clear that these modifications affect both microtubule dynamics and interactions with microtubule associated proteins (MAPS) in cells, and therefore serve as control elements in a variety of biological processes.

Tubulin polyglutamylation occurs at the C-termini of both α- and β-tubulin.[4–7] This typically involves the addition of one to six additional glutamate residues, and the overall extent of tubulin polyglutamylation increases during development.[8–11] The first glutamate is added to the side chain of a main chain glutamate to form an isopeptide bond in a process called initiation (Fig. 1). Subsequent glutamate residues could conceivably be added to either the α-carboxylate or the γ-carboxylate in elongation steps. HPLC analyses using synthetic peptides have indicated that α-elongations predominantly occur during human brain tubulin polyglutamylation.[8,10,11] These PTM's are catalyzed by a series of ATP-dependent amino acid ligases that are members of the 'tubulin–tyrosine ligase-like' (TTLL) family of enzymes.[6] These enzymes belong to the ATP-grasp family of ligases that include the prototypical member D-alanine–D-alanine ligase as well as tubulin–tyrosine ligase (TTL).[12,13] Of the 13 known TTLL enzymes in the human genome, ten have been implicated as glutamylases.[2] In vitro studies using recombinant enzyme have only been performed on one of these, TTLL7, and it has been reported that this enzyme is capable of catalyzing both initiation and elongation.[14] As mentioned previously, this PTM is reversible and the enzymes that remove the glutamate residues from tubulin have recently been identified as members of the soluble cytosolic carboxypeptidase (CCP) family.[15,16] Four CCP members have been implicated as tubulin deglutamylases; however, in vitro activity has not yet been demonstrated for most of them.

Polyglutamylation has been shown to regulate the activity of the microtubule associated molecular motors kinesin and dynein.[3,17,18] Not surprisingly, polyglutamylating enzymes are crucial for normal neuronal development.[19,5] Tubulin polyglutamylation has also been implicated in positively regulating the activity of the microtubule severing enzyme spastin,[20] a protein that is mutated in more than 40% of patients diagnosed with hereditary spastic paraplegias.[21] Loss of spastin function has been implicated in defects in mitosis,[22] late stage cytokinesis events,[23] as well as dendritic arborization.[24] Moreover, it has been found that prostate and pancreatic cancer cells display higher levels of polyglutamylation than normal cells.[25,26] In particular, a recent study showed that TTLL4 is highly expressed in pancreatic cancer cells and knockdown of TTLL4 attenuated their growth,[25] supporting the idea of using TTLL enzymes as therapeutic targets for small molecule

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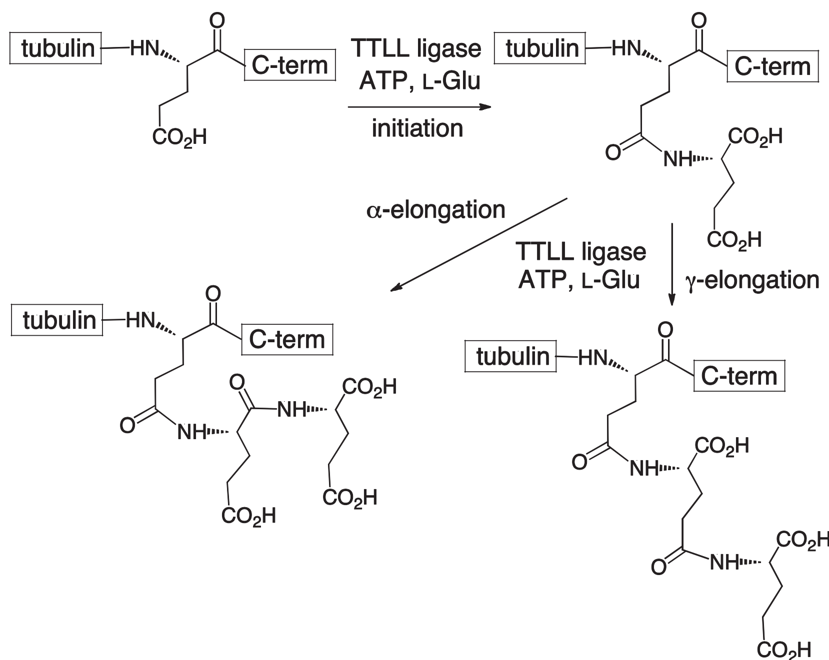

Figure 1. The initiation and elongation steps of tubulin polyglutamylation catalyzed by the TTLL enzymes.

inhibitors. Furthermore, hyperglutamylation has been linked to neurodegeneration in mouse models and inhibition of the TTLL1 polyglutamylase reversed this neurodegenerative phenotype.[15] Thus, potent inhibitors of the tubulin polyglutamylation cycle could play key roles in understanding the structure and function of these enzymes and could serve as lead compounds in the development of therapies based on interfering with tubulin PTM levels.

Phosphinic acids are known to serve as effective inhibitors of both ATP-dependent ligases and carboxypeptidases.[27–38] The tetrahedral geometry and negative charge serves as an excellent mimic of the tetrahedral intermediate formed in the ligase reaction (Fig. 2). In some instances, the bound phosphinic acid may be phosphorylated to generate a tightly bound phosphorylated inhibitor.[13,33–35] The potency of these inhibitors is often quite impressive and K_I values in the low nanomolar range have been reported for both the ligases and the carboxypeptidases.[27,28,32,36,37] In order to target the tubulin glutamylation enzymes, three phosphinic acid inhibitors were designed (Fig. 3). Inhibitor **1** is expected to mimic the tetrahedral intermediate formed in the initiation step and inhibitors **2** and **3** are expected to mimic the intermediates formed in the α- and γ-elongation steps, respectively. These compounds are expected to serve as inhibitors of the corresponding deglutamylation enzymes as well. In this study we describe the synthesis of inhibitors **1**–**3** and their inhibition of the TTLL7 reaction using a tubulin tail peptide as substrate.

Inhibitors **1** and **3** resemble compounds previously prepared for the inhibition of folylpoly-γ-glutamate handling-enzymes and were synthesized in an analogous fashion.[39,27] The synthesis of inhibitor **1** began with the treatment of the known alkene (*S*)-**4** with ammonium hypophosphite and triethylborane to give the mono-alkylphosphinic acid (Scheme 1).[39,40] This acid was silylated and added to dimethyl 2-methylenepentanedioate to generate the di-alkylphosphinic acid.[41,42] This was immediately methylated to give compound **5** as a mixture of four diastereomers. The Cbz and benzyl groups were removed by hydrogenolysis and the resulting amino acid was acetylated and coupled to ethylamine to give compound **6**. Demethylation was performed with NaOH to give inhibitor **1** as a mixture of two diastereomers. The

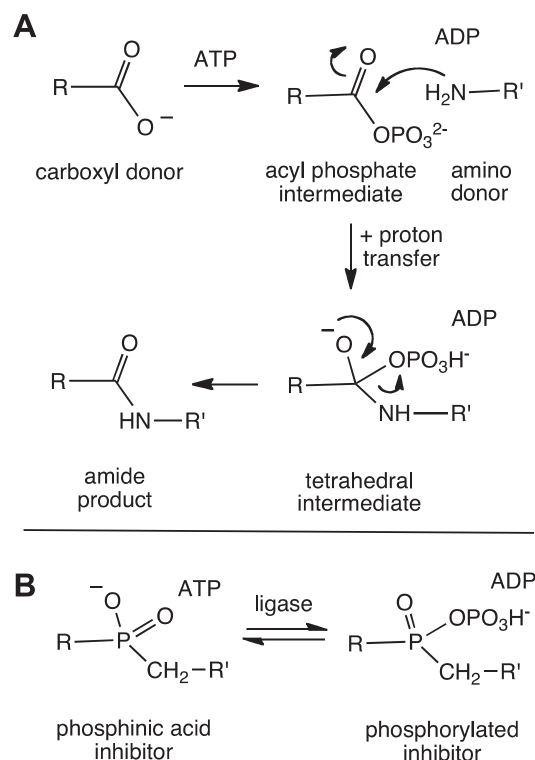

Figure 2. (A) The mechanism employed by the ATP-dependent amino acid ligases. (B) The structure of phosphinic acid-based inhibitors and the potential phosphorylation of the inhibitor within the ligase active site.

precursor to inhibitor **3**, compound **7**, was also prepared by the hydrogenolysis of compound **5** followed by acetylation and methylation. Global demethylation using NaOH produced inhibitor **3** as a mixture of two diastereomers. In order to prepare inhibitor **2**, a strategy reported by Georgiadis et al. was employed in which a phenyl group is used as a carboxyl synthon (Scheme 2).[41] The

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