Di-, tri- and tetra-5'-O-phosphorothioadenosyl substituted polyols as inhibitors of Fhit: Importance of the α-β bridging oxygen and β phosphorus replacement

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Abstract

Background: The human FHIT gene is inactivated early in the development of many human cancers and loss of Fhit in mouse predisposes to cancer while reintroduction of FHIT suppresses tumor formation via induction of apoptosis. Fhit protein, a diadenosine polyphosphate hydrolase, does not require hydrolase activity to function in tumor suppression and may signal for apoptosis as an enzyme-substrate complex. Thus, high affinity nonhydrolyzable substrate analogs may either promote or antagonize Fhit function, depending on their features, in Fhit + cells. Previously synthesized analogs with phosphorothioadenosyl substitutions and "supercharged" branches do not bind better than natural substrates and thus have limited potential as cellular probes.

Results: Here we link adenosine 5'-O-phosphates and phosphorothioates to short-chain polyols to generate a series of substrate analogs. We obtain structure-activity data in the form of in vitro Fhit inhibition for four types of analog substitutions and describe two compounds, inhibitory constants for which are 65 and 75-fold lower than natural substrates.

Conclusions: The best Fhit inhibitors obtained to date separate two or more 5'-O-phosphoromonomothioadenosyl moieties with as many bond lengths as in ApppA, maintain oxygen at the location of the α-β bridging oxygen, and replace carbon for the β phosphorus.

Background

Loss of Fhit protein is among the earliest known events in the development of a variety of the most common and lethal human malignancies [1]. Loss of Fhit leads to cells that are deficient in programmed cell death and that form tumors in mice while Fhit reexpression in Fhit-cancer cells reduces tumorigenicity and restores pro-
AppppA (1) into AMP plus ADP and ATP, respectively [8–10]. His96, which is responsible for covalent catalysis and more than $4 \times 10^6$-fold of rate enhancement in AppppA hydrolysis [9,11–13], is nonetheless dispensable for AppppA-binding and tumor suppression, suggesting that Fhit function in tumor suppression depends on formation of an E-S complex [2,12]. If Fhit-substrate complexes promote tumor suppression by stimulating a pro-apoptotic effector, then Fhit inhibitors that resemble natural substrates may promote Fhit function. Similarly, Fhit inhibitors with normative features may antagonize Fhit function. Either class of compounds may be important in dissecting Fhit cell biology and regulating apoptosis.

Making use of a synthesis strategy to link adenosine 5'-O-phosphates and phosphorothioates to short-chain polyols [14], we evaluate four inhibitor parameters and obtain compounds, inhibitory constants for which are as much as 70-fold lower than natural substrates. Key features of the best candidate agonist compound 6b and candidate antagonist compound 12b are conservation of the length of the polyphosphate replacement, use of 5'-O-phosphorothioadenosyl residues, no replacement for oxygen at the location of the \( \alpha-\beta \) bridging oxygen, and replacement of carbon for the \( \beta \) phosphorus. Compound 12b contains additional negatively charged substituents that may facilitate Fhit inhibition [15] while rendering the compound antagonistic to Fhit function in the cell. Covalent structures of AppppA (1) and compounds 6b and 12b are provided in Figure 1.

**Results and Discussion**

A series of compounds 2–6 were synthesized and evaluated for Fhit inhibition that link two 5'-O-phosphorothioadenosyl or AMP groups with five reagents ranging in size from ethylene glycol to meso-erythritol. Sodium salts of each compound were titrated into assays of purified Fhit [16] with 1.8 \( \mu \)M fluorescent substrate, Appp-S-(4-4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacine-3-yl)methylaminoacetyl (ApppBODIPY) [10], and competitive \( K_i \) values were obtained by calculating the inhibitor concentration-dependence in reduction of \( k_{cat}/K_m \) (apparent) for substrate hydrolysis [10].

As shown in Table 1, inhibitors 2b and 3b with two-carbon diol linkers were either only as inhibitory as the natural substrate 1 or substantially less so. Inhibitor 4b with a four-carbon linker was as ineffective as 3b. Inhibitors 5a and 6a that substitute a central CH\(_2\)-PO\(_2\)-CH\(_2\) or CH\(_2\)-CH(OH)-CH\(_2\) for the PO\(_2\)-O-PO\(_2\) of 1 were as inhibitory as 1 is a good substrate. The phosphorothioate analogs, 5b and 6b, bound approximately 10 and 75 times better to Fhit than 1. Thus, conservation of bond-lengths between adenylate moieties of 1, 5 and 6 is conducive to binding Fhit.

Because phosphorothioate analogs of 5 and 6 were better inhibitors than the corresponding phosphates, phosphorodithioate analog 6c was prepared together with five additional compounds as phosphates and phosphorothioates. Phosphorodithioate 6c was a less effective inhibitor (\( K_i = 8600 \) nM, not shown in Tables) than corresponding phosphate 6a and phosphorothioate 6b and, as demonstrated in Tables 1 and 2, the phosphorothioate congener of every compound made as a phosphate and a phosphorothioate had a lower \( K_i \) value. The contribution of particular P-chiral phosphorothioate stereoisomers to inhibition has not been examined. When diadenosine 5',5''-(P\(_1\), P\(_2\)-methylene-P3-thio)-P\(_1\), P3-triphosphate[17] was crystallized with wild-type and mutant Fhit, the \( \alpha \)-phosphorothioate group was found in the mutant but not wild-type active site, suggesting that \( \alpha \)-phosphorothioate inhibitors may be slow substrates...
Similarly, Frey and co-workers found that Fhit slowly cleaves both $R_p$ and $S_p$ stereoisomers of $\gamma$-(m-nitrobenzyl)-adenosine 5'-O-([l-thiotriphosphate) with modest and similar beneficial effects on $K_m$ as compared to the corresponding phosphate [13].

Given the inhibitor activity of 6b, the contribution of the oxygen in the position of the $\alpha$-$\beta$ bridging oxygen of 1 was examined. As can be seen in Table 2, comparison of 9b with 10b and 11b and of 15b with 16b indicated that imido or sulfur substitutions for oxygen reduce enzyme inhibition by an order of magnitude. Derivatives of 6b with

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Table 1: Fhit inhibition by AppppA analogs varied in interadenylate linker and phosphorothiolation.

| Comp. | RI | $X = O$ $K_i$ (nM) | $X = S$ $K_i$ (nM) |
|-------|----|------------------|------------------|
| 1     |    | 2600             | -                |
| 2     |    | -                | 2600             |
| 3     |    | -                | 86000            |
| 4     |    | -                | 86000            |
| 5     |    | 1500             | 220              |
| 6     |    | 4700             | 35               |
one or two additional functionalities on the central carbon were characterized. Analysis of 9b indicated that loss of the polar hydroxyl group reduces inhibitory activity and analysis of 7b and 8b indicated that nearly isoelectronic groups that are nonisoelectronic are not tolerated. The magnitude of these effects was surprising and may be related to altered conformations upon manganese coordination. Earlier, Blackburn and co-workers made "supercharged" methane-trisphosphonic acid AppppA analogs containing a central carbon from which three adenylate or phosphate moieties are bonded [15]. In work presented here, tripodal inhibitors 15a and 17a showed, respectively, micromolar and submicromolar efficacies while the phosphorothioate counterparts 15b and 17b showed 100 nM efficacy.

Derivatives of pentaerythritol, tetrapodal compounds 12,13 and 14, were also evaluated for Fhit inhibition. While compound 14b, containing two phosphorothiolated branches without adenosine, was barely a submicromolar inhibitor, compounds 12b and 13b, which contain respectively three and four CH₃-phosphorothioadenosyl groups bonded to the central carbon were 40 nM and 65 nM inhibitors. Thus, while the simplest compound 6b was initially rendered less inhibitory by modification because its central hydroxyl was important for inhibition, addition of one or two CH₃-phosphorothioadenosyl groups restored inhibition. We presume that compounds 6b, 12b and 13b with 2, 3 or 4 identical CH₃-phosphorothioadenosyl groups can present a manganese bound 6b-like complex to Fhit in a similar manner. On the basis that Fhit binds diadenosine polyphosphates with one AMP group buried and the other adenosine solvent-exposed in a specific conformation [12], effective tri and tetrapodal inhibitors such as 12b, 13b and HC(ADP)₃ [15] may exist in solution predominantly in a conformation that resembles Fhit-bound AppppA.

On the basis of observations [12,13] discussed above, one would expect AppppA with two phosphorothioadenosyl substituents to be a slow substrate with a relatively low $K_m$. However, carbon in place of the β phosphorus (for example in 6b) would make the leaving group the alkoxide – OCH₃-CH(OH)-CH₂O-phosphorothioadenosyl rather than α-thio ATP. Presumably, because normal Fhit products are mononucleotides with low $pK_a$ values, the enzyme does not have a group to protonate an alkoxide leaving group and thus substitution of carbon for the β phosphorus turns a substrate into an inhibitor.

### Table 2: Fhit inhibition by AppppA analogs varied in phosphorothiolation and three modifications of glycerol.

| Comp | R₁ | R₂       | R₃       | $X = O$ $K_i$ (nM) | $X = S$ $K_i$ (nM) |
|------|----|----------|----------|------------------|------------------|
| 6    | O  | OH       | H        | 4700             | 35               |
| 7    | O  | CH₃      | H        | -                | 76000            |
| 8    | O  | NH₃      | H        | -                | 3000             |
| 9    | O  | H        | H        | -                | 230              |
| 10   | NH | H        | H        | -                | 3300             |
| 11   | S  | H        | H        | -                | 5200             |
| 12   | O  | -CH₂-O-P-(X,O)-OAdo | -CH₂-O-P-(X,O₂) | 1500             | 40               |
| 13   | O  | -CH₂-O-P-(X,O)-OAdo | -CH₂-O-P-(X,O)-OAdo & | 3200             | 65               |
| 14   | O  | -CH₂-O-P-(X,O₂) | -CH₂-O-P-(X,O₂) | 900              | 700              |
| 15   | O  | -O-P-(X,O)-OAdo | H        | 1500             | 78               |
| 16   | NH | -O-P-(X,O)-OAdo | H        | -                | 2700             |
| 17   | O  | -O-P-(X,O₂) | H        | 420              | 110              |
Conclusions
Tumor suppression by Fhit is not destroyed by mutation of the nucleophilic His96 to Asn, a mutation that specifically reduces \( k_{\text{cat}} \) [2,12]. Thus, evidence suggests that the proapoptotic function of Fhit depends on formation of an E-S complex. If the substrate-dependent signaling model is correct, then compound 6b, as a nearly isosteric AppppA analog with a low \( K_i \), may promote Fhit signaling in Fhit+ cells. Compound 12b, which one would expect to bind Fhit with bulky phosphorothioate and phosphorothioadenosyl groups interfering with putative effector binding, may consequently prove to be antagonistic to Fhit function. Ongoing in vitro studies aim to evaluate the stereochemistry of phosphorothiol binding to Fhit while in vivo studies test the effects of these compounds on programmed cell death. Phosphorothioate analogs 6b and 12b that preserve the \( \alpha-\beta \) bridging oxygen but substitute carbon for the \( \beta \) phosphorus, achieving 65 to 75-fold binding advantages over AppppA, are expected to be critical for cell biological characterization.

Materials and Methods
Synthesis and Characterization
The synthesis and physicochemical characteristics of compounds 5, 6b and b, 12 through 15, and 17 have been described [14]. Compounds 2–5, 8b, 9b were obtained in the reaction of two equivalents of \( 5'-\text{O-(2-thiono-1,3,2-oxathiaphospholane)}-\text{N}^6,\text{N}^6,\text{O}^2',\text{O}^3'\)-tetra benzoyladenosine (18) with one equivalent of ethylene glycol (for 2b); \( \text{O,O-dimethyl-D,L-tartrate} \) (for 3b); \( \text{meso-erythritol} \) (for 4b); \( \text{O-methyl bis(hydroxymethyl)phosphinate} \) (for 5); 2-amino-1,3-propanediol (for 8b); 1,3-propanediol (for 9b). To prepare compound 6c, two equivalents of \( 5'-\text{O-(2-thiono-1,3,2-dithiaphospholane)}-\text{N}^6,\text{N}^6,\text{O}^2',\text{O}^3'\)-tetra benzoyladenosine (19) were condensed with one equivalent of glycerol. Compounds 7b, 10b, 11b, 16b were obtained in the reaction of \( \text{N}^6,\text{N}^6,\text{O}^2',\text{O}^3'\)-tetra benzoyladenosine with 2-thiono-1,3,2-oxathiaphospholane derivatives of 2-methyl-1,3-propanediol (for 7b); 1,3-diaminopropane (for 10b); 1,3-propanedithiol (for 11b) and 1,3-diamino-2-hydroxypropene (for 16b). Compounds 12 through 14 were obtained in the reaction of tetra-oxathiaphosphothiolated erythritol (20) with two equivalents of \( \text{N}^6,\text{N}^6,\text{O}^2',\text{O}^3'\)-tetra benzoyladenosine (21) for 12; three equivalents of 21 for 13; four equivalents of 21 for 14. Compound 15 was obtained in the reaction of tri-oxathiaphosphothiolated glycerol (22) with three equivalents of compound 21 [14].

Synthesis of \( 5'-\text{O-(2-thiono-1,3,2-oxathiaphospholane)-N}^6,\text{N}^6,\text{O}^2',\text{O}^3'\)-tetra benzoyladenosine (18)
\( \text{N}^6,\text{N}^6,\text{O}^2',\text{O}^3'\)-tetra benzoyladenosine (1 mmol) was reacted with 2-chloro-1,3,2-oxathiaphospholane (1.1 mmol) in pyridine solution (3 ml) in the presence of elemental sulfur (5 mmol). After stirring for 12 h at room temperature, the solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography using chloroform:hexane (8:2) as an eluent to provide 18 in 72% yield \([31^\text{P NMR 105.2 ppm (d), FAB-MS (M-1) m/z 820}]\).

Synthesis of \( 5'-\text{O-(2-thiono-1,3,2-dithiaphospholane)-N}^6,\text{N}^6,\text{O}^2',\text{O}^3'\)-tetra benzoyladenosine (19)
\( \text{N}^6,\text{N}^6,\text{O}^2',\text{O}^3'\)-tetra benzoyladenosine (1 mmol) was reacted with 2-chloro-1,3,2-dithiaphospholane [18] in pyridine solution (3 ml) in the presence of elemental sulfur (5 mmol). After stirring for 12 h at room temperature, the solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography using chloroform:hexane (8:2) as an eluent to give 19 in 68% yield \([31^\text{P NMR 115.2 ppm, FAB-MS (M-1) m/z 836}]\).

Condensation of oxathiaphospholane derivatives with polyols
A mixture of 1,8-diazabicyclo(5,4,0) undec-7-ene under 7-ene with the corresponding polyol (one equivalent of 1,8-diazabicyclo(5,4,0) undec-7-ene per -OH function) in 1 ml acetonitrile solution was added to the solution of one molar equivalent of oxathiaphospholane derivative (18,19,20 or 21) dissolved in dry acetonitrile (5 ml). The reaction mixture was stirred at room temperature for 4 hours and then solvent was removed under reduced pressure. Purified compounds were obtained by Sephadex A-25 ion-exchange chromatography using a linear gradient of ammonium bicarbonate buffer (pH 7.5) as eluent, and their physicochemical characteristics are given in Table 3.

Enzyme inhibition assays
Inhibitors, at approximately 4,2, 1, 0.5 and 0.25 times \( K_i \) value, were added to assays of Fhit with AppppBODIPY [10]. \( K_i \) values were obtained by calculating the inhibitor concentration-dependence in reduction of \( k_{\text{cat}}/K_m \) (apparent) as earlier described [10].
Table 3: Physicochemical characteristics of compounds 2–17.

| Comp. No | H$_3$PO$_4$ chemical shifts [ppm] | MS-MALDI (M-1) m/z [per cent] | Yield [%] |
|----------|---------------------------------|-------------------------------|-----------|
| 2        | 58.95; 57.64                    | 752                           | 24        |
| 3        | 58.82; 57.69                    | 840                           | 53        |
| 4        | 57.14; 56.92                    | 812                           | 50        |
| 5*       | 58.46; 58.24; 30.28             | 815                           | 54        |
| 6a*      | 2.3                             | 750                           | 35        |
| 6b*      | 56.83                           | 782                           | 42        |
| 6c       | 115.86; 115.159                 | 814                           | 25        |
| 7b       | 57.64; 57.33                    | 780                           | 21        |
| 8b       | 59.13; 58.94                    | 781                           | 10        |
| 9b       | 57.32; 58.15                    | 766                           | 34        |
| 10b      | 60.65; 60.31                    | 764                           | 55        |
| 11b      | 71.85; 71.17                    | 798                           | 40        |
| 12*      | 56.94; 46.82                    | 1267                          | 20        |
| 13*      | 56.28                           | 1516                          | 35        |
| 14*      | 57.12; 46.71                    | 1018                          | 21        |
| 15*      | 57.56                           | 1127                          | 60        |
| 16b      | 60.17; 59.13; 58.49; 57.41      | 1125                          | 15        |
| 17*      | 56.61; 45.95                    | 877                           | 37        |

*Compounds described in ref. [14].

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