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Research article

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

James M Varnum[1], Janina Baraniak[2], Renata Kaczmarek[2], Wojciech J Stec[2] and Charles Brenner*[1]

Address: [1]Structural Biology & Bioinformatics Program, Kimmel Cancer Center, Philadelphia, USA and [2]Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Lodz, Poland

E-mail: James M Varnum - varnum@dada.jci.tju.edu; Janina Baraniak - baraniak@bio.cbmm.lodz.pl; Renata Kaczmarek - kacmarek@bio.cbmm.lodz.pl; Wojciech J Stec - wistec@bio.cbmm.lodz.pl; Charles Brenner* - brenner@dada.jci.tju.edu

*Corresponding author

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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*-phosphoromonothioadenosyl moieties with as many bond lengths as in AppppA, 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 programmed cell death [2–6]. Infection of *Fhit* +/- mice with viruses that re-express Fhit reduce cancer occurrence [7], apparently by killing pre-neoplastic cells that have lost the wild-type *Fhit* allele. Human Fhit protein, a member of the Fhit branch of the histidine triad superfamily of nucleotide-binding proteins, binds and hydrolyzes diadenosine polyphosphates such as ApppA and

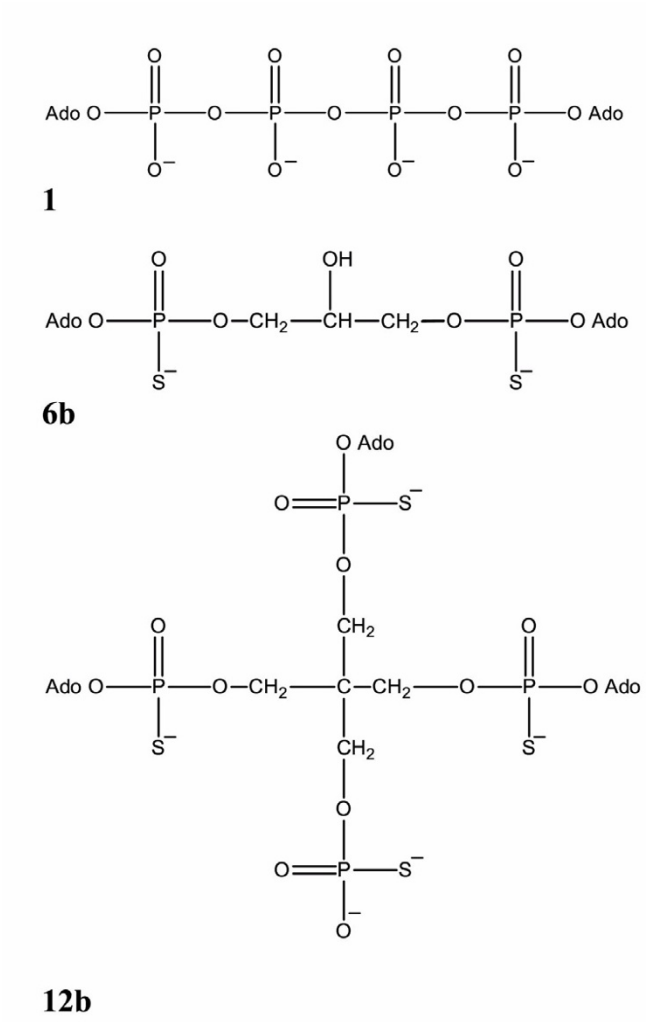


Figure 1
Structures of Fhit substrate 1 and Fhit inhibitors 6b and 12b.

AppppA (**1**) into AMP plus ADP and ATP, respectively [8–10]. His96, which is responsible for covalent catalysis and more than 4×10^6-fold of rate enhancement in AppA hydrolysis [9,11–13], is nonetheless dispensable for ApppA-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.

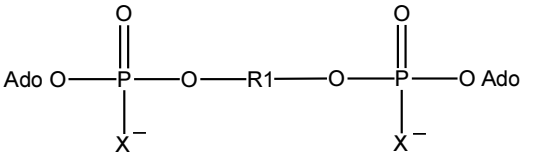
Making use of a synthesis strategy to link adenosine 5'-*O*-phosphates and phosphorothioates to short-chain polyols [14], we evaluate four inhibitor parameters and obtain compounds, inhibitory constants for which are as much as 70-fold lower than natural substrates. Key features of the best candidate 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 α-β bridging oxygen, and replacement of carbon for the β 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.

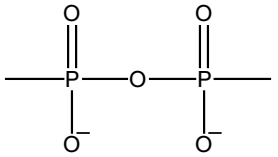
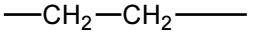
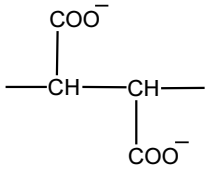
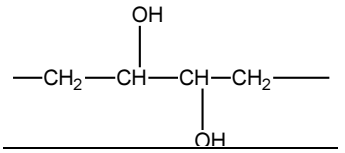
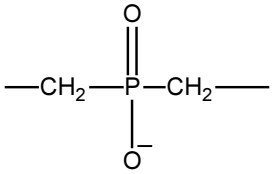
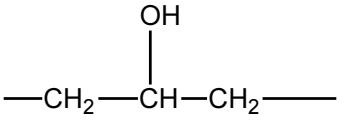
Results and Discussion

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 μM 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-P^3-thio)-P^1, P^3-triphosphate[17] was crystallized with wild-type and mutant Fhit, the α-phosphorothioate group was found in the mutant but not wild-type active site, suggesting that α-phosphorothioate inhibitors may be slow substrates

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

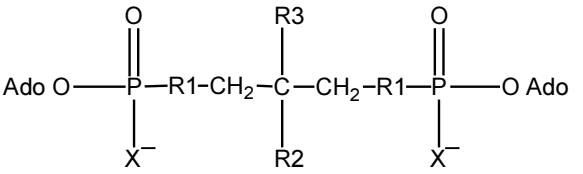


Comp.	R1	X = O K_i (nM) a	X = S K_i (nM) b
1		2600	-
2		-	2600
3		-	86000
4		-	86000
5		1500	220
6		4700	35

[12]. Similarly, Frey and co-workers found that Fhit slowly cleaves both R_p and S_p stereoisomers of γ-(*m*-nitrobenzyl)-adenosine 5'-*O*-(1-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 α-β 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

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



Comp	R1	R2	R3	X = O K_i (nm) a	X = S K_i (nM) b
6	O	OH	H	4700	35
7	O	CH_3	H	-	76000
8	O	NH_3	H	-	3000
9	O	H	H	-	230
10	NH	H	H	-	3300
11	S	H	H	-	5200
12	O	-CH_2-O-P-(X,O)-OAdo	-CH_2-O-P-(X,O_2)	1500	40
13	O	-CH_2-O-P-(X,O)-OAdo	-CH_2-O-P-(X,O)-OAdo	3200	65
14	O	-CH_2O-P-(X,O_2)	-CH_2-O-P-(X,O_2)	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,O_2)	H	420	110

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 isosteric 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 phosphorothioylated branches without adenosine, was barely a submicromolar inhibitor, compounds **12b** and **13b,** which contain respectively three and four CH_2-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 CH_2-phosphorothioadenosyl groups restored inhibition. We presume that compounds **6b, 12b** and **13b** with 2, 3 or 4 identical CH_2-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 – OCH_2-CH(OH)-CH_2O-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.

Conclusions

Tumor suppression by Fhit is not destroyed by mutation of the nucleophilic His96 to Asn, a mutation that specifically reduces k_{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 phosphorothioyl 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 α-β bridging oxygen but substitute carbon for the β 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, 6a** 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'-*O*-(2-thiono-1,3,2-oxathiaphospholane)-N^6,N^6,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine (**18**) with one equivalent of ethylene glycol (for **2b**); *O,O*-dimethyl-D,L-tartrate (for **3b**); *meso*-erythritol (for **4b**); *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'-*O*-(2-thiono-1,3,2-dithiaphospholane)-N^6,N^6,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine (**19**) were condensed with one equivalent of glycerol. Compounds **7b, 10b, 11b, 16b** were obtained in the reaction of N^6,N^6,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine with 2-thiono-1,3,2-oxathiaphospholane derivatives of 2-methyl-1,3-propanediol (for **7b**); 1,3-diaminopropane (for **10 b**); 1,3-propanedithiol (for **11b**) and l,3-diamino-2-hydroxypropane (for **16b**). Compounds **12** through **14** were obtained in the reaction of tetra-oxathiaphosphothioylated erythritol (**20**) with two equivalents of -N^6,N^6,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine (**21**) for **12;** three equivalents of **21** for **13;** four equivalents of **21** for **14.** Compound **15** was obtained in the reaction of tri-oxathiaphosphothioylated glycerol (**22**) with three equivalents of compound **21** [14].

*Synthesis of 5'-O-(2-thiono-1, 3, 2-oxathiaphospholane)-N^6,N^6,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine (**18**)*

N^6,N^6,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine (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}P NMR 105.2 ppm (d), FAB-MS (M-1) m/z 820].

*Synthesis of 5'-O-(2-thiono-1,3,2-dithiaphospholane)-N^6,N^6,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine (**19**)*

N^6,N^6,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine (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}P NMR 115.2 ppm, FAB-MS (M-1) m/z 836].

*Synthesis of tetra-oxathiaphosphothioylated erythritol (**20**) or tri-oxathiaphosphothioylated glycerol (**21**)*

2-chloro-1,3,2-oxathiaphospholane (8 mmol) was added to a suspension of elemental sulfur (10 mmol) in pyridine (10 ml). A solution of glycerol (1.5 mmol) or erythritol (1.5 mmol) in pyridine (1 ml) was then introduced to the reaction mixture and stirred for 12 h at room temperature. Crude products were purified by silica gel column chromatography using chloroform:hexane (7:3) to give **22** in 77% yield [^{31}P NMR 105.6 ppm (m), FAB-MS (M-l) m/z 505] or **21** in 60% yield [^{31}P NMR 105.1 ppm (m), FAB-MS (M-1) m/z 687], respectively.

Condensation of oxathiaphospholane derivatives with polyols

A mixture of 1,8-diazabicyclo(5,4,0) undec-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 mixtures were 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 ApppBODIPY [10]. K_i values were obtained by calculating the inhibitor concentration-dependence in reduction of k_{cat}/K_m (apparent) as earlier described [10].

Table 3 : Physicochemical characteristics of compounds 2–17.

Comp. No	^{31}P NMR in D_2O H_3PO_4 chemical shifts [ppm]	MS-MALDI (M-1) m/z	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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