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Evaluation of influence of Ap_4A analogues on Fhit-positive HEK293T cells; cytotoxicity and ability to induce apoptosis

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ABSTRACT

Fragile histidine triad (Fhit) protein encoded by tumour suppressor *FHIT* gene is a proapoptotic protein with diadenosine polyphosphate (Ap_nA, $n = 2–6$) hydrolase activity. It has been hypothesised that formation of Fhit–substrate complex results in an apoptosis initiation signal while subsequent hydrolysis of Ap_nA terminates this action. A series of Ap_nA analogues have been identified in vitro as strong Fhit ligands [Varnum, J. M.; Baraniak, J.; Kaczmarek, R.; Stec, W. J.; Brenner, C. *BMC Chem. Biol.* **2001**, *1*, 3]. We assumed that in Fhit-positive cells these compounds might preferentially bind to Fhit and inhibit its hydrolytic activity what would prolong the lifetime of apoptosis initiation signalling complex. Therefore, several Fhit inhibitors were tested for their cytotoxicity and ability to induce apoptosis in Fhit-positive HEK293T cells. These experiments have shown that Ap_4A analogue, containing a glycerol residue instead of the central pyrophosphate and two terminal phosphorothioates [A_{PS}-$CH_2CH(OH)CH_2$-$_{PS}$A (**1**)], is the most cytotoxic among test compounds ($IC_{50} = 17.5 \pm 4.2$ μM) and triggers caspase-dependent cell apoptosis. The Fhit-negative HEK293T cells (in which Fhit was silenced by RNAi) were not sensitive to compound **1**. These results indicate that the Ap_4A analogue **1** induces Fhit-dependent apoptosis and therefore, it can be considered as a drug candidate for anticancer therapy in Fhit-positive cancer cells and in Fhit-negative cancer cells, in which re-expression of Fhit was accomplished by gene therapy.

1. Introduction

Diadenosine polyphosphates (Ap_nA), ($n = 2–6$) act as intra- and extracellular signalling molecules, the level of which regulates numerous physiological processes in the cell.[1] Ap_nA molecules are also substrates for hydrolytic activity of Fhit protein.[2] Fhit is a tumour suppressor protein involved in various cellular processes including cell cycle control, sensitivity to DNA-damaging agents and induction of apoptosis.[3–5] Fhit protein is encoded by the fragile histidine triad (*FHIT*) gene, which is frequently inactivated in human tumours and precancerous lesions.[6,7] Deletion or inactivation of *FHIT* results in either complete loss or decreased expression of the Fhit protein, respectively. Re-expression of *FHIT* gene in Fhit-negative tumour cells significantly inhibits growth and increases apoptosis in vitro,[8–10] as well as suppresses the tumourigenicity of these cells in vivo.[11]

Fhit is an enzyme (EC 3.6.1.29) that catalyses the cleavage of the α-phosphate bond in diadenosine polyphosphates (Ap_nA), with Ap_3A and Ap_4A being its major substrates.[2,12] Interestingly, it is believed that the hydrolytic activity of Fhit is not related to its tumour suppressor function.[13] The H96N Fhit mutant, which lacks hydrolytic activity but effectively binds the substrate, is functional as a tumour suppressor in vitro.

The pro-apoptotic activity of Fhit correlates with its substrate binding affinity.[14] Fhit-negative tumour cells expressing exogenous Fhit-mutants with a 2–7-fold increased K_m (lower affinity) are less effective at promoting cell death than wild-type *FHIT*-infected cells. Furthermore, cells expressing a mutant Fhit with a threefold higher K_m show low levels of apoptosis, similar to cells without the *FHIT* gene. Therefore, the Fhit–substrate complex is thought to be a signalling molecule for one or more pro-apoptotic pathways.[13,14] According to this hypothesis, the Fhit dimer surface becomes filled with two Ap_nA molecules, and this complex serves as a possible site of effector interactions. Hydrolysis of the substrate terminates signal transduction, suggesting that the lifetime of the signalling molecule is key for long-term signalling.[13] The molecular details and the binding partners involved in this mechanism have not been fully elucidated yet. It has been shown that hUbc9 (human ubiquitin conjugating enzyme of sumoylation system) binds to Fhit protein and suppresses its hydrolase activity. This suppression may elongate the lifetime of the Fhit–Ap_3A signalling complex, influencing on the antitumour activity of Fhit.[15] Other reports demonstrate that Fhit interacts with tubulin in a manner independent of Ap_3A hydrolase activity,[16] but the connection between tumour suppression and

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binding to tubulin is poorly understood. However, the interaction of Fhit with hUbc9 and tubulin has not been confirmed by others.[17] On the other hand, the Fhit–substrate complex interacts with Fdxr (ferredoxin reductase) and heat shock proteins, and the mitochondrial localisation of these proteins is important for Fhit tumour suppressor function.[18] Moreover, Hela cells die upon exogenous introduction of Fhit and simultaneous treatment with protoporphyrin IX (PpIX).[19] It is suggested that this effect is connected with the formation of a complex consisting of PpIX, Fhit and either Ap_3A-substrate or AMP-product of the Fhit hydrolytic action, and a stabilized three-member complex serves as an apoptosis signal causing cell death.[20] Concluding, the role of Fhit hydrolytic activity in apoptosis signalling remains unclear.

The aim of our study was to evaluate an influence of a series of Ap_4A analogues **1–5** of general formula ApsxpsA (where x is a substituted glycerol residue, and PS is a phosphorothioate linkage, Fig. 1), on Fhit-positive HEK293T cells. These compounds were reported to exhibit high affinity for Fhit protein under in vitro conditions and to act as inhibitors of Fhit—hydrolytic activity (K_i value from 35 to 100 nM).[21] The cytotoxicity of compounds **1–5** as well as their ability to induce apoptosis in Fhit-positive HEK293T cells and in Fhit-negative cells, in which *FHIT* gene was silenced by RNAi were evaluated by MTT and annexin V, caspase-3, -8 and -9 assays. We assumed that compounds which are able to induce Fhit-dependent cell death might be good drug candidates for anti-cancer therapy in Fhit-positive cancer cells and in Fhit-negative cells in which Fhit expression is induced by transgene technology.

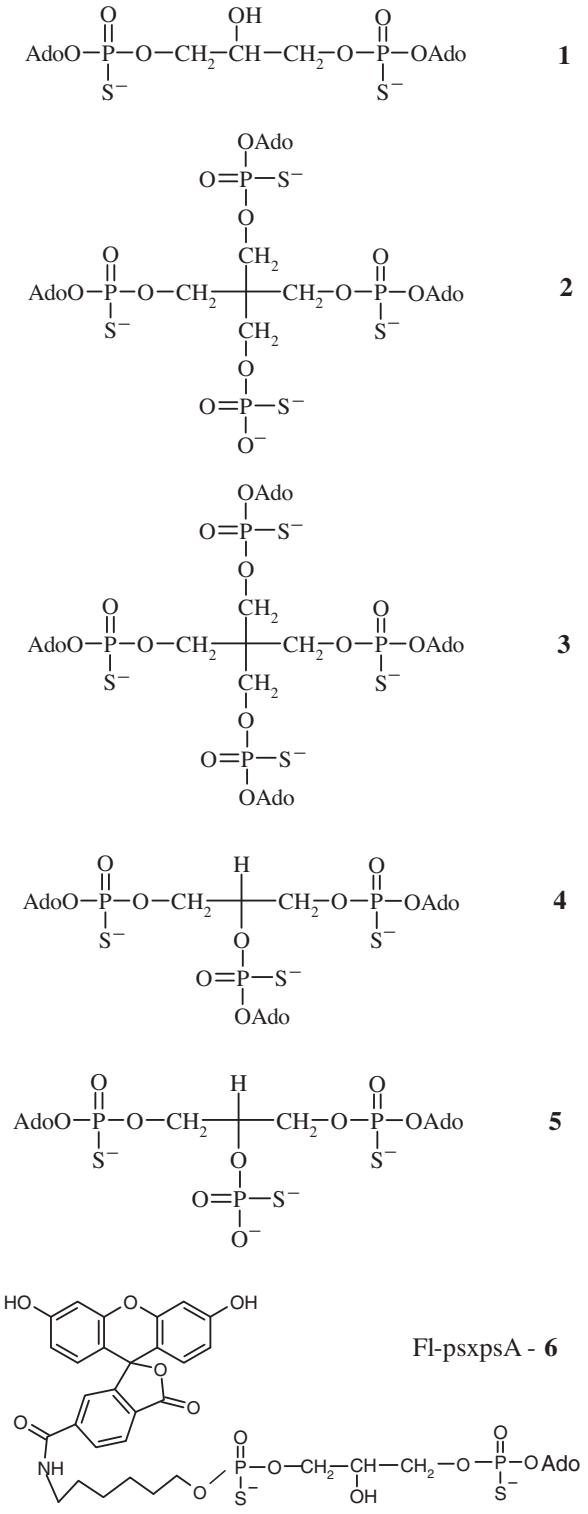

Figure 1. Structures of ApsxpsA **1–6** analogues. Compounds were used as a mixture of diastereoisomers.

2. Results

2.1. Stability of compounds 1–5 in cellular medium and in cellular extract

The stability of compounds **1–5** was determined in medium collected from culture of HEK293T cell. This medium contained serum as well as some membrane enzymes released by the cells. In these conditions, after 48 h of incubation all tested compounds **1–5** remained intact at more than 97% (1 mM concentration of **1–5**, incubation up to 48 h, 37 °C), while Ap_3A and Ap_4A were degraded at 95%.

The Ap_3A, Ap_4A and ApsxpsA-**1** stability experiments performed in HEK293T cellular extract, containing a total protein lysate with native, active enzymes, showed that compound **1** remained untouched at more than 90% after 48 h, whereas Ap_3A and Ap_4A were almost completely hydrolysed (at level 88.7% and 92.8%, respectively).

2.2. Cytotoxicity of compounds 1–5

To assess the toxicity of compounds **1–5** towards Fhit-positive HEK293T cells, cell viability was measured after 24, 48 and 72 h by the MTT assay.[22] In this assay a yellow tetrazole salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT] is reduced by mitochondrial reductase to purple formazan, which can be quantified spectrophotometrically. The absorbance of formazan is

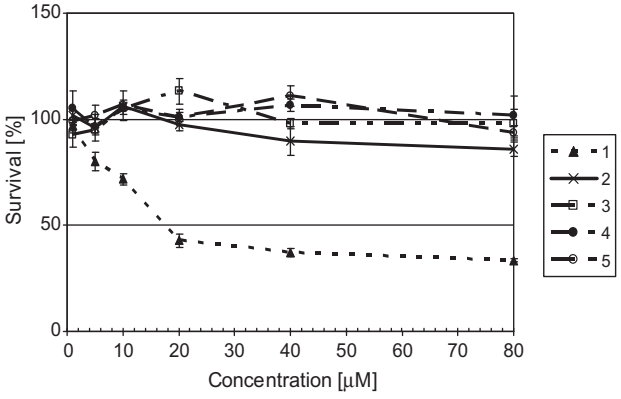

Figure 2. HEK293T cell viability measured by MTT assay after 72 h incubation with various concentrations of ApsxpsA (**1-5**). IC_{50} = 17.5 ± 4.2 μM for molecule **1** was determined.

relative to the number of living cells. These measurements have shown that compounds **2**–**5** exhibit low cytotoxicity (IC_{50} >80 μM), while compound **1** is remarkably cytotoxic with an IC_{50} of 17.5 ± 4.2 μM after 72 h (Fig. 2). Therefore, subsequent experiments were performed with the most cytotoxic compound **1** only.

The cytotoxicity of **1** was much higher in Fhit-positive HEK293T cells and cancer HL-60 cell line (IC_{50} = 9 ± 1.4 μM, 72 h) than in A549 and HeLa Fhit-negative cancer cells, where the IC_{50} was above 80 μM after 72 h of incubation. The Fhit-protein status was confirmed in all studied cell lines by western blotting followed by immunostaining with Fhit-specific antibodies (Supplementary data, Fig. S1).

2.3. Caspase assays

2.3.1. Caspase-3

To verify the presumed correlation between cytotoxicity of 1 and the level of apoptosis in HEK293T cells, the activity of caspase-3 was measured in a fluorescent assay.

This enzyme is an effector caspase and a marker of two main apoptotic pathways, namely the extrinsic and the intrinsic pathways.[23,24] Activity of caspase-3 depends on many factors including time of incubation, cell line and type, and concentration of inducing agent. Therefore, various amounts of **1** were used, and measurements were performed at several time points. Figure 3 shows changes in the activity of caspase-3 in HEK293T cells after incubation with compound **1**. The most significant increase in the caspase 3 activity (approximately 27%, p <0.05) was observed after 18 h of incubation at the 5 μM concentration of the inducer (Fig. 3a). The observed changes were concentration-dependent, as there were 10% and 17% increases in caspase-3 activity when the concentration of **1** was raised from 0.5 to 1.0 μM and from 0.5 to 5.0 μM, respectively (p <0.05) (Fig 3b). There was virtually no change in the level of apoptosis when the cells were treated with 1 at concentrations ranging from 5 to 10 μM.

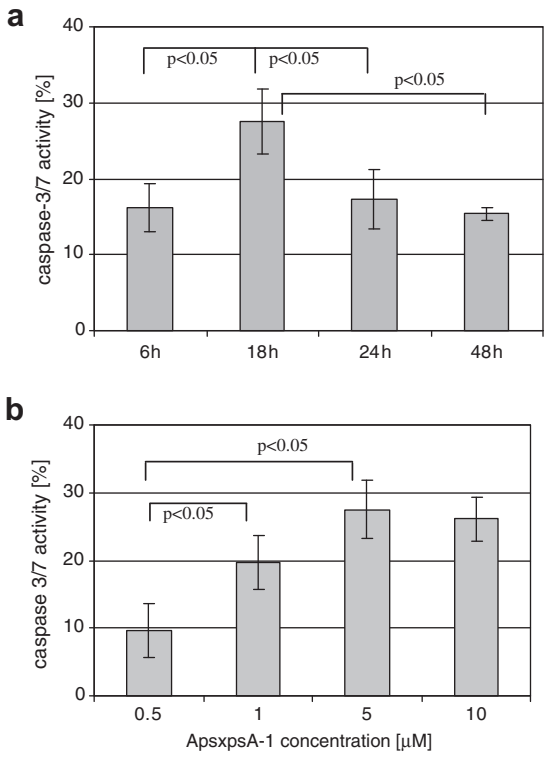

Figure 3. Caspase 3 activity measured in HEK293T cells treated with Ap_sxp_sA-**1**. (a) 5 μM concentration, incubation time: 6 h, 18 h, 24 h and 48 h. (b) 0.5, 1.0, 5.0, 10 μM concentration and incubation time 18 h.

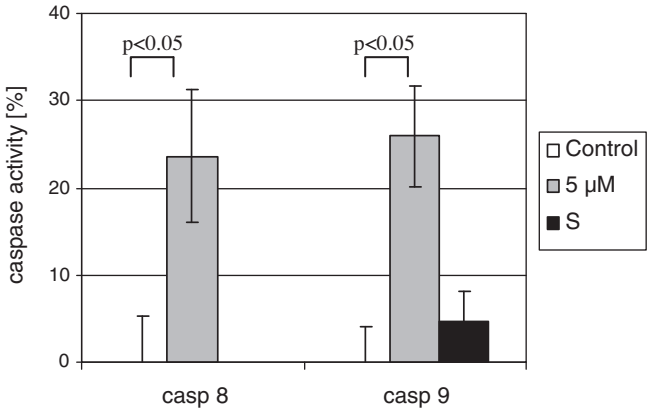

Figure 4. Caspase 8 and caspase 9 activity after 18 h incubation with Ap_sxp_sA-**1** at 5 μM concentration. Staurosporine (0.5 μM) was used as the positive control.

2.3.2. Caspases-8 and -9

To investigate which apoptotic pathway was elicited by 1, we measured the level of caspase-8 and caspase-9 in the cells by luminescent assay. We found that both of these caspases, 8 and 9, were activated in the Fhit-positive HEK293T cells (Fig. 4). After 18 h of incubation with compound 1 at 5 μM (the concentration producing the most significant effect in the caspase-3 assay), we observed a 23% increase in caspase-8 activity (p <0.05) and 21% increase in caspase-9 activity (p <0.05) as compared to the non-activated cells.

2.3.3. Caspase-3 assay after treatment of HEK293T cells by Fhit-specific siRNA

To obtain Fhit-negative control cells, we generated HEK293T cells in which *FHIT* expression had been substantially down-regulated by using Fhit-specific RNAi. Two different siRNAs (Table 1) were designed, both containing wobble base pair (G:U) at the 5′-end of the duplexes (in the respect to the antisense strand). Such a non-canonical base-paring introduces thermodynamic asymmetry into the duplex, and if it is placed as described above, it can enhance the activity of siRNA.[25]

As shown in Figure 5a, silencing of Fhit was performed effectively, as the Fhit protein level in the siRNA-2-treated cells 48 h after transfection was decreased by 69% (expression of Fhit was 31% only) compared to that of the cells transfected with the non-specific siRNA used as a control. The experiment performed on HEK293T cells with the decreased *FHIT* expression, which were additionally incubated for 18 h with **1** (5 μM) has shown that the caspase-3 activity was not detectable (p <0.05), (Fig. 5b). In cells transfected with non-specific siRNA and compound **1**, the level of caspase-3 activity was similar to that of control cells (treated with studied compounds and lipofectamine only). Moreover, similar

Table 1
The sequences of siRNA molecules: sense (S) and antisense strands (AS) sequences are given

siRNA	Sequence
siRNA-1	S 5′-gcccucuguaguguuucu**u**aa-3′ AS 3′-uucgggagacaucacaaaga**g**-5′
siRNA-2	S 5′-guggccgauuuguuucaga**u**ga-3′ AS 3′-uucaccggcuaaacaaagucu**g**-5′
Control siRNA	S 5′-aaucagauugaaccuucautt- 3′ AS 3′-ttuuagucuaacuuggaagua-5′

Wobble base pair are marked in bold.

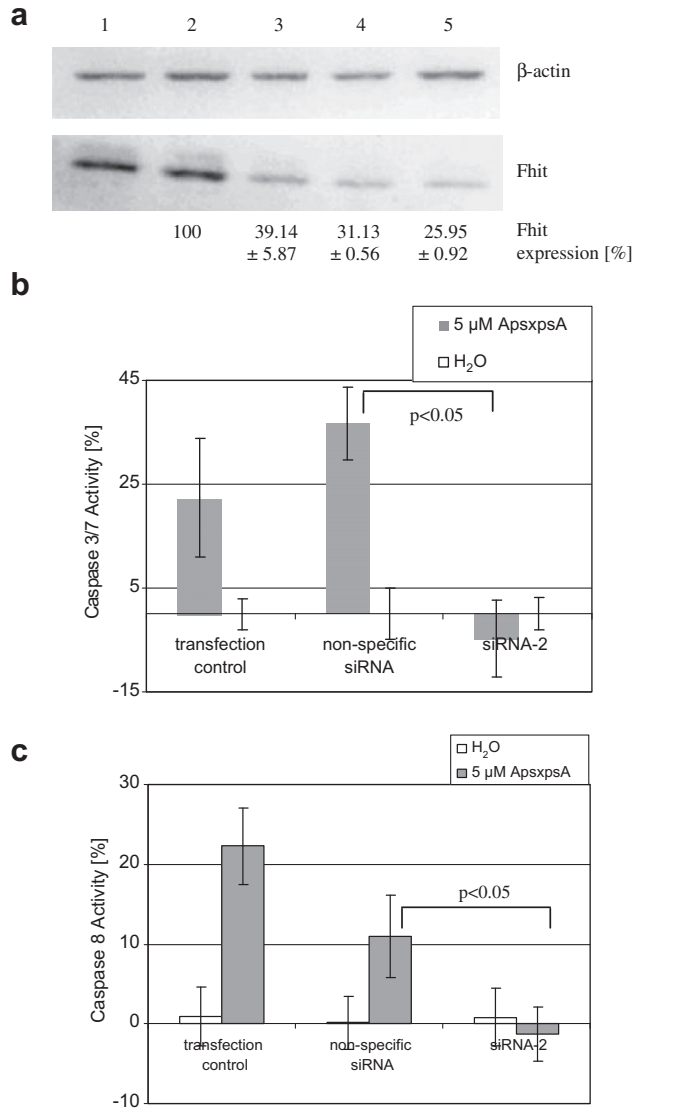

Figure 5. Silencing of the FHIT gene expression in HEK293T cells by the use of FHIT mRNA specific RNA interference. (a) Western blot analysis (48 h after transfection) of the Fhit protein in cells treated with Lipofectamine2000 (control cells, lane 1); transfected with the control non-specific siRNA (lane 2); and with siRNA-1 (lane 3); siRNA-2 (lane 4) and a mixture (lane 5). (b) Caspase-3 and (c) caspase-8 activity measured 48 h after treatment of the HEK293T cells with siRNA-2 and after additional 18 h of incubation with ApsxpsA-**1** (5 μM). As controls, cells treated with Lipofectamine2000 (transfection control) and transfected with the non-specific siRNA were used.

experiment, where caspase-8 level was measured (Fig. 5c) also revealed lack of activity of this protease (p <0.05) in comparison to control cells.

2.4. Annexin-V assay

To confirm the results of the assays for caspases-3, -8 and -9, we have used a second method for visualisation of apoptosis by means of detection of changes in the position of phosphatidyl serine in the cell membrane.[26] This apoptosis assay was performed by fluorescence microscopy of the living cells stained with annexin-V-FITC conjugate (green-stained cells) and propidium iodide dye (red-stained cells). The data for apoptotic cells with exposed phosphatidyl serine (green-stained and green + red-stained cells) were collected as described in experimental section. At 24 h, control, untreated HEK293T cells showed the presence of 3.9 ± 0.95% apoptotic cells. After treatment with **1** (20 μM) 12.27 % ± 1.03 apoptotic cells were observed. At a lower concentration of **1** (5 μM), phosphatidyl serine was also exposed but at levels 10.5 ± 1.5% and 14.24 ± 0.64% after 24 h and 48 h upon treatment, respectively. Comparison of untreated HEK293T cells with the cells treated with compound **1** demonstrates that molecule **1** is able to induce cell apoptosis. These results confirm data obtained from caspase assays.

2.5. Cellular uptake of the ApsxpsA-1

To study the cellular uptake of ApnA analogues in a cell, a fluorescent analogue of the diadenosine tetraphosphate, compound **6** was synthesised. Fl-psxpsA-**6** is structurally related to the ApsxpsA-**1** molecule but contains one fluorescein moiety instead of an adenosine residue (Fig. 1). Experiments were performed without any carrier, only by the incubation with 7.5 μM concentrations of **6**. After 24 h compound **6** was present inside the HEK293T cells (Fig. 6a, b), demonstrating that molecules containing a glycerol residue instead of one central phosphate moiety are able to penetrate into cells without any additional agents. In all experiments, the fluorescent probe **6** had been localised within cytoplasmic compartments and vesicular structures and was visible as fluorescent particles. On the other hand, fluorescein alone (7.5 μM), used as a control, was visible as equal distributed in the entire cell and showed different arrangement in the cells than molecule **6** (Fig. 6c and d).

3. Discussion

Naturally occurring dinucleoside polyphosphates affect a variety of biological activities in a wide range of target tissues.[27] Ap_nA (n = 2–6) are known to act as signalling molecules and many physiological and pathophysiological processes are correlated with the change of their cellular level.[1] More metabolically stable Ap_nA analogues, as compared to naturally occurring nucleotides, represent effectory molecules for enzymes with Ap_nA substrate specificity. From a series of reported Ap_nA analogues,[21,28] we chose compounds **1**–**5** of the general formula ApsxpsA, where x is a substituted glycerol residue and PS is a phosphorothioate linkage (Fig. 1) for evaluation of their influence on Fhit-positive HEK293T cells. These compounds, sharing several structural features common to the Ap_3A and Ap_4A, were stable in HEK293T cell culture medium and in the HEK293T cellular extract.

The MTT screening assay demonstrated that among the five studied compounds, only analogue **1** was cytotoxic (IC_{50} = 17.5 μM after 72 h), whereas analogues **2**–**5** showed low toxicity (IC_{50} >80 μM) in HEK293T Fhit-positive cells. It is worth noting that, in contrast to analogues **2**–**5**, compound **1** is an isosteric Ap_4A analogue. The molecules **2**–**5** are spatially larger in comparison to compound **1**, as they contain an additional phosphorothioate moiety or bulky phosphorothioadenosyl groups (Fig. 1).

To evaluate the possible mechanism by which the most cytotoxic compound **1** induces cell death in HEK293T cells, we measured the activity of the apoptotic indicators: caspases-3, 8, and -9. Our results demonstrate that molecule **1** is able to induce cell apoptosis by activation of all three caspases (Figs. 3 and 4). These findings also suggest that apoptotic pathways, the extrinsic and the intrinsic (by activating caspase-8 and caspase-9, respectively) are induced by compound **1**. Additional apoptotic fluorescent assay, in which the cells were stained with annexinV-FITC conjugate and propidium iodide, confirmed that compound **1** was able to trigger apoptosis.

One may consider induction of apoptosis by activation of P2 type receptors by adenosine nucleotides.[29,30] Native HEK293 cells express endogenous P2Y receptors, of the $P2Y_1$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$

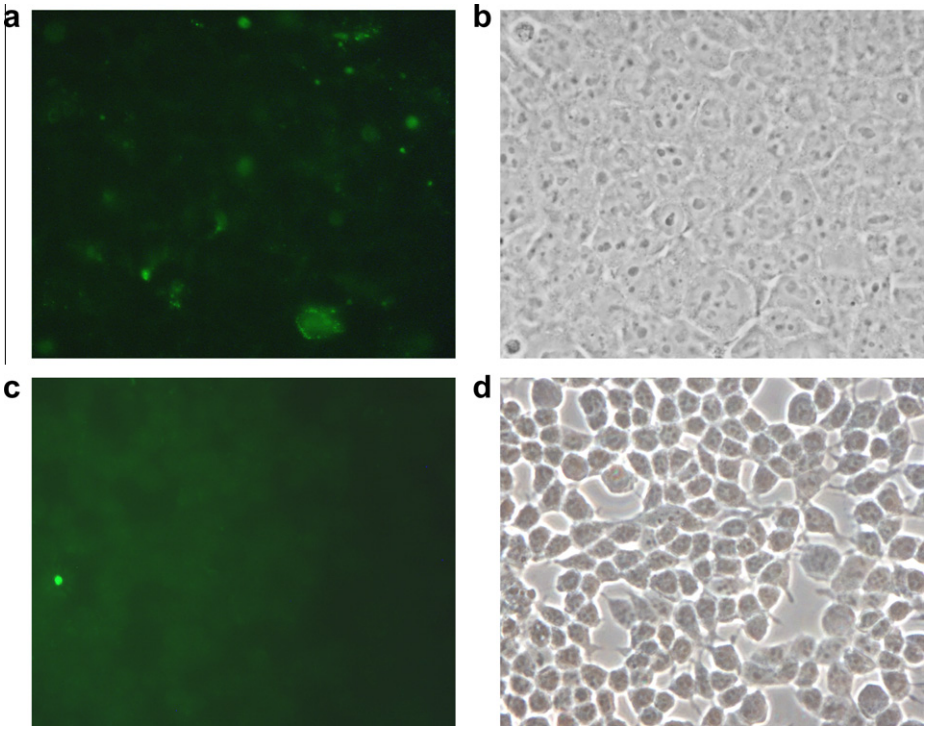

Figure 6. Microscopic imaging of cellular uptake of Fl-p_sxp_sA-**6** into the HEK293T cells after incubation without carrier (7.5 μM, 24 h) - presented as fluorescence (a) and phase contrast (b) to demonstrate efficiency of the transport. Fluorescein (c-florescence and d-phase contrast) was used as control.

and $P2Y_{13}$ subtypes,[31] but do not possess P2X receptors.[31–33] Ap_3A and Ap_4A were described as agonists of $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors.[29] The only $P2Y_2$ was demonstrated to be able to trigger cell death by apoptosis in human oesophageal cancer cells,[34] although $P2Y_1$ receptors can influence on cancer cells by decreasing cell proliferation in melanoma[35] and $P2Y_{11}$ can influence on differentiation effects in leukaemia cells.[36] Activation of apoptosis through $P2Y_2$ occurs after prolonged incubation with ATP at minimum 50 μM concentration but not with ATPγS suggesting that ecto-ATPase depending generation of extracellular P_i is involved in this apoptotic process.[35]

On the other hand, we found that molecule **6**, fluorescent analogue of **1**, is present inside the HEK 293T cells after 24 h of incubation at 7.5 μM concentration without any carrier (Fig. 6).

Due to these findings, there is a very low probability that the apoptosis induced by compound **1** (used at 5 μM concentration, even at 17 μM) was generated externally in HEK293T cells. Moreover, analogues **2**–**5**, which contain adenosine residues, similarly to compound **1** (Fig. 1), do not cause any death of HEK293T cells (MTT assay, Fig. 2) in contrary to the molecule **1**. If external receptor activation could occur then it should not be any difference in the MTT assay among the derivatives **1**–**5** and all of them should demonstrate the cytotoxicity on similar, noticeable level, what is not a case.

Concerning the mechanism of intracellular action of compound **1**, the interaction with tumour suppressor Fhit was taken into account because of high affinity of molecule **1** for this protein (K_i value 35 nM[21]). As it was demonstrated (Fig. 4), ApsxpsA-**1** activates caspase-8 and -9. It has been shown that Fhit protein induces apoptosis by activation of the caspase cascade[14] and triggers the extrinsic, and the intrinsic apoptotic pathways, thereby activating both caspase-8[37,38] and caspase-9.[10,39]

Previous studies (see Section 1) hypothesised that the active Fhit signalling complex consists of the Fhit protein and the Ap_nA substrate and that hydrolysis of the substrate's polyphosphate chain terminates the apoptotic signal.[13,14] Consequently, the use of stable Ap_nA analogues instead of the Ap_nA substrate, is expected to prolong the lifetime of the signalling complex and to keep the apoptosis pathway active, as we demonstrated above. On the other hand, in agreement with the hypothesis, in the Fhit deficient cells apoptosis should not be induced after treatment by analogue **1**. We found that in Fhit-silenced HEK293T cells (69% reduction of Fhit expression by RNAi, Fig 5a) we did not observe caspase-3 activity after incubation with **1** at 5 μM concentration (Fig. 5b). This result univocally indicates a lack of apoptosis in the cells deprived of the Fhit protein. Decreased Fhit expression to about 30% is sufficient to have the significant effect as above and we suggest three possible reasons for this result. First, likelihood of Fhit–substrate complex formation decreases when deficiency of one of the complex members occurs. Second, effectors cannot bind efficiently to suboptimal levels of the Fhit–substrate complex concentration. For example, the Fhit–substrate/Fxdr/Hsp60 complex does not form and transport of these proteins to mitochondria is interrupted, interfering with the induction of apoptosis. Third, the signal to induce apoptosis may not be strong enough because of low concentration of the signalling molecule. Concluding, lowering Fhit concentration to suboptimal level seems to have important implications for Fhit-dependent apoptosis.

There is some evidence in the literature that Ap_4A may constitute the nucleotide component of the signalling Fhit–substrate complex.[40]It has been suggested that an increased level of Ap_4A or an increased Ap_4A/Ap_3A ratio induces apoptosis in certain tumour cells after treatment with etoposide, while Ap_3A levels increase during cell differentiation.[41] On the other hand, apoptosis is associated with an elevated Ap_3A level or Ap_3A/Ap_4A ratio in Fhit-positive HEK293 cells exposed to stress conditions (i.e., etoposide, Cd2+, anti-Fas antibody, sorbitol or cold shock)[42] and only after hyperosmotic shock the degree of apoptosis was correlated with Ap_4A, but not Ap_3A levels. A very low level of intracellular Ap_3A is observed in normal conditions in Fhit-positive cells,

perhaps due to Fhit hydrolytic activity.[43] However, if some signal-mediated modifications appear and are able to reduce the rate of hydrolysis, Ap_3A would accumulate,[42] and the likelihood of Fhit–Ap_3A complex formation would increase. Adding exogenous Ap_3A or an isosteric compound like ApsxpsA-**1** to Fhit-positive cells should also increase probability of the signalling complex formation. Moreover, in contrary to Ap_3A, analogue **1** is not hydrolysed, so the lifetime of signalling molecule should be prolonged and the apoptosis effect should be more significant.

Crystallographic studies have shown that the binding of an analogue of Ap_3A, in which one of the P–O–P residues is substituted with P–CH_2–P by the hydrolytically inactive Fhit H96N mutant is virtually identical to that of the wild-type Fhit.[13] It has also been shown that the conformation of Fhit does not change significantly in the Fhit–substrate complex after substrate binding and does not depend on the identity of associated ligands (AMP, Ap_3A or Ap_4A).[13,44]

4. Conclusion

Our screening results (MTT assay, Fig. 2) showed that not all studied Ap_4A analogues are able to trigger cell death in HEK293T, Fhit-positive cells. Although the K_i = 1.8 μM (the value close to K_m for Ap_3A/Fhit) that we observed for the ApsxpsA-**1**/Fhit complex is slightly different from that reported in the literature,[21] this difference does not alter our conclusions.

After excluding the possibility that apoptosis was caused by compound **1** acting externally on the cells, the intracellular interactions with Fhit protein were taken into account. The ApsxpsA-**1** molecule, a near isosteric Ap_4A analogue, is able to pass across the cell membrane and to act as an agonist of the presumed substrate, and activate the caspase-dependent apoptosis pathway in Fhit-positive cells by promoting Fhit signalling. Compounds **2**–**5**, which were expected to bind Fhit and interfere with putative effector binding, do not force cell death (MTT assay), possibly because they act as antagonists to Fhit functioning. Moreover, induction of caspase-dependent apoptosis after treatment of Fhit-positive HEK293T cells with molecule **1**, and on the other hand, lack of apoptosis after administering **1** to Fhit-silenced HEK293T cells, indicate that compound **1** induces apoptosis in Fhit-dependent manner probably by forming signalling complex with this protein. As a result, the lifetime of the signalling complex could be prolonged what promotes apoptosis as it has been previously suggested.[13]

The lack of toxic effect of **1** in Fhit-negative cancer cell lines (A549 and Hela) as well as the high IC_{50} value of **1** used at micromolar concentrations in Fhit-positive cancer cell line (HL-60) also support this hypothesis.

In our studies, the ApsxpsA-**1** congener was used as a mixture of diastereoisomers (P-chiral centres of the Rp–Rp; Sp–Sp and Rp–Sp configuration). Therefore, it would be interesting to compare the affinity of each separate diastereomer for the Fhit enzyme, as well as to assess the ability of each compound to activate the caspase cascade. Studies on this topic are in progress in our laboratory.

Although it has been reported that only one of the three P-chiral diastereoisomers of **1** (Rp–Rp) was resistant towards *sv*pde nuclease at specific conditions,[45] our data have shown that in cellular conditions stability of the diastereomeric mixture **1** is high. The observed effects of this study suggest that dinucleoside polyphosphates can be substituted by specific, stable isosteric analogues, which in turn may become candidate drugs for Fhit transgene therapy of Fhit-negative cells or in some Fhit-positive cancer cells. In this regard, comparing other paired Fhit+ and Fhit− cancer cell lines after treatment with compound **1** is important and, thus, additional studies are in progress.

5. Materials and methods

5.1. Chemical synthesis

Compounds **1**–**5** (Fig. 1) were obtained as described previously.[21] Their structure was confirmed spectrally by MALDI-TOFF MS and ^1H and ^{13}C NMR (data not shown). Compounds **1**–**5** used for these studies were the mixtures of diastereoisomers at P-chiral centre of phosphorothioate functions.

For the synthesis of the fluorescent probe **6** (an analogue of **1** carrying a fluorescein moiety) 1,3-*O*,*O*-di-DMT-glicerol attached through the 2-OH group to the solid support (typical CPG) was used (loading 28 μmol/g). The monomers, prepared in house adenosine-2′,3′-*O*,*O*-N^6-tribenzoyl-5′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl-phosphoramidite (30 mg) and commercially available fluorescein phosphoramidite (Glen Research, Sterling, Virginia USA) (30 mg) were mixed, dried overnight under high vacuum at room temperature and dissolved in 3 ml of anhydrous acetonitrile (Fluka). The solution was introduced onto an automatic 6-columns DNA/RNA synthesiser GeneWorld (K&A, Germany). The solid support (125 mg, 3.5 μmol) was evenly distributed into 6 reaction columns (0.2 μmol) and the DMT groups were removed in a regular way (3% DCA in methylene chloride, 45 s). 1H-Tetrazole promoted condensation was carried over 600 s, followed by sulfurisation (Beaucage reagent, 300 s). The content of all six columns was combined, the products were released from the solid support by treatment with concentrated ammonia (2 ml) and the benzoyl groups were then removed at 55 °C for 12 h. After evaporation at reduced pressure, the desired product was isolated by means of RP-HPLC in the amount of 9 OD units. Its structure was confirmed by MALDI-TOF MS (m/z 990). The presence of two absorbance maxima were detected at UV–vis spectrum (at 260 nm and 494 nm).

5.2. Fhit enzyme inhibition assay

A K_i value of **1** to Fhit was obtained by calculating the inhibitory concentration-dependent in reduction of k_{cat}/K_m (apparent) as described earlier.[12,21]

5.3. Cell line

All cell lines were grown at 37 °C in an atmosphere of 5% CO_2 with addition of 100 U/L penicillin G and 100 U/L streptomycin in following mediums: HEK293T (human embrionic kidney) cells in Dulbecco's modified Eagle's medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% foetal bovine serum (FBS) (Gibco, BRL, Paisley); Hela (cervical cancer) and A549 (human lung carcinoma) in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO) with 10% FBS and HL-60 cells (promyelocytic leukaemia) in RPMI 1640 medium with 20% FBS.

5.4. Stability of ApsxpsA in cellular medium and cellular extract

Substrates (ApsxpsA **1**–**5**) used at 1 mM concentration were incubated at 37 °C with the medium collected from HEK293T cell cultures. Reaction samples (⩽2 μl) were spotted on TLC Silica Gel 60 F_{254} plates (Merck) after the appropriate incubation times (6, 18, 24 and 48 h). Plates were air-dried and developed in propanol-2/NH_4OH/H_2O (7:5:1), imaged by epi-short wave UV illumination and quantified in a G-Box (SynGene, Cambridge, England) instrument using GeneTools 4.00 software.

HEK293T cellular extract was prepared from cell culture of a 75 ml flask with cells grown to confluence. Cells were scraped, sonicated in PBS buffer with Complete®, a mixture of protease inhibitors (Roche, Penzberg, Germany). The samples were centrifuged

and the clear supernatant was used as total protein lysate. For stability experiments, 40 μg of total protein lysate and ApsxpsA-**1** at 1 mM concentration were incubated at 37 °C in PBS buffer. The reaction was monitored as above after incubation at 48 h.

5.5. Cell viability test

HEK 293T (human embryonic kidney 293 cells with the SV40 large T-antigen) cells in the exponential growth phase were seeded in complete medium at a concentration of 10×10^3 per 200 μl and were allowed to adhere for 16–24 h. Tested compounds ApsxpsA (**1**–**5**, Fig. 1), were added at the final concentrations of 1.0, 5.0, 10, 20, 40 and 80 μM and the number of cells was determined after 24 h, 48 h and 72 h by means of the tetrazolium salt method (MTT assay).[22] Each MTT experimental point represents the mean ± SE from at least two independent measurements performed in quadruplicate. MTT assay for HL-60, A549 and HeLa cells were performed in similar way.

5.6. Silencing of the FHIT gene in HEK293T cells by siRNAs

On the basis of literature,[46] siRNA-1 and siRNA-2 corresponding to FHIT nt393–413 and FHIT nt523–544, respectively, were selected as the target sequences for RNA interference (RNAi). The sequences of the synthetic RNA molecules and their complements are listed in Table 1. RNA oligonucleotides were synthesised and purified by a method described previously,[47] and their molecular weights were confirmed by MALDI-TOF mass spectrometry. siRNA duplexes were prepared by mixing equimolar amounts of complementary sense- and antisense RNA strands (final duplex concentration was 20 μM), heating at 95 °C for 2 min, followed by slow cooling to room temperature over 3 h.

Cells were transfected with the siRNAs duplexes (siRNA-1, siRNA-2 and the mixture of siRNA-1 and siRNA-2) at 100 nM concentration by the method using Lipofectamine2000 Transfection reagent (Invitrogen) in triplicate in 6-well plates (5 μl of lipofectamine/well). The efficiency of the RNAi action was evaluated 48 h after transfection by western blot analysis. Nonspecific siRNA was used as a control.[48]

5.7. Western blot analysis

Samples were sonicated in PBS buffer containing COMPLETE Protease inhibitor cocktail (Roche). Total protein (20 μg) from each sample was separated on a 4%/15% polyacrylamide gel and electroblotted to a PVDF membrane (Millipore). The filter was blocked in 5% non-fat dry milk, incubated with a rabbit primary antibody [against human Fhit (Abcam) or β-actin (Zymed)], washed, probed with goat anti-rabbit IgG conjugated to alkaline–phosphatase (Zymed) and developed with Immobilon Western Chemiluminescence AP Substrate (Millipore). Membranes were visualised using a G-Box (SynGene, Cambridge, England) instrument with chemiluminescent camera and analysed by GeneTools 4.00 software.

5.8. Caspase-3 and -8, -9 activity analysis

Caspases activation was measured using the Apo-ONE homogeneous caspase-3/7 assay, CaspaseGlo-8 assay or CaspaseGlo-9 assay, according to the manufacturer's instructions (Promega, USA). Briefly, cells in the exponential growth phase were seeded in 96-well black/white plates (30×10^3 HEK293T cells/well) and were allowed to adhere for 16–24 h. Then compound **1** (0.5, 1.0, 5.0 and 10 μM for caspase3/7 assay and 5.0 μM of compound **1** for caspase 8 and 9 assay were added. After the appropriate time (6, 18, 24 or 48 h for caspase-3 and 18 h for caspases 8 and 9), cell lysis was performed by incubation at room temperature in the dark with the lysis buffer and the caspase substrate. Finally, fluorescence (caspase-3) or luminescence (caspase-8 or caspase-9) values were measured using a Synergy HT plate reader (BIO-TEK). The amount of fluorescence/luminescence detected as relative light units was proportional to caspase activity and was calculated with the following equation: [(caspase activity of the probe − caspase activity of the control)/caspase activity of the control] × 100%. Non-treated cells were used as the control. Data points represent the mean ± SE from at least two measurements performed in triplicate. Statistical analysis was performed using one-way ANOVA or *t*-test and data with $p \leqslant 0.05$ were assigned as statistically significant.

Caspase-3 and caspase-8 assays with the FHIT-knockdown HEK293T cells, which were obtained 48 h after siRNA-2 transfection, were performed after 18 h incubation of 5 μM of compound **1**. The cells treated with water instead of the tested compounds (for transfection control, nonspecific siRNA and siRNA-2) were used as controls and the calculations were preformed as above.

5.9. Annexin V assay

For live-cell fluorescence microscopy, the HEK293T cells were cultured in 96-well plates in 0.2 ml of medium and treated with ApsxpsA-**1** at 20 μM concentration. After 24 h, annexin-V conjugated with fluorescein isothiocyanate (FITC) (1/100 dilution, BD Biosciences) and propidium iodide (5 μg/ml, Sigma) were added to medium and cells were visualised by fluorescence microscopy using a Nikon Eclipse T*i*-U inverted microscope. FITC was excited with the 465/495 nm filter and detected using a 515/555 nm filter. PI fluorescence was excited with 540/525 nm and detected using a 605/655 nm long pass filter. Images were taken for three fields chosen per well with a mean number of 200 ± 31 cells per field (the 20x zoom, 0.34 μm = 1 px). Analysis of the images was carried out using NIS element basic Software. Data for annexin-V-FITC (green) and propidium iodide (red) binding were correlated to count apoptotic events occurring within the analysis. Apoptotic cells were defined as those that fluoresced green or green and red and calculated as the number of events per total cells present in the studied image x 100%.

5.10. Cellular up-take of Fl-psxpsA-6

Cells were cultured overnight in 8-well plates (adhered to the glass slides) at a density of 70×10^3 cells/well in complete medium. Next, the compound **6** or fluorescein were added to the medium to the final concentration of 7.5 μM (the volume of the concentrated solution supplement do not exceed 1%). After the 24 h of incubation, the cells were washed three times with a phosphate saline buffer (PBS), fixed with 3.8 % paraformaldehyde for 15 min at room temperature and again washed three times with PBS. Then, a cover slip was placed on the slide using DAPCO/glycerol mounting medium. The slides were visualised by fluorescence microscopy using a 515/555 nm filter.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.06.028.

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