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Small-Molecule Inhibitors of the Tumor Suppressor Fhit

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The tumor suppressor Fhit and its substrate diadenosine triphosphate (Ap_3A) are important factors in cancer development and progression. Fhit has Ap_3A hydrolase activity and cleaves Ap_3A into adenosine monophosphate (AMP) and adenosine diphosphate (ADP); this is believed to terminate Fhit-mediated signaling. How the catalytic activity of Fhit is regulated and how the Fhit·Ap_3A complex might exert its growth-suppressive function remain to be discovered. Small-molecule inhibitors of the enzymatic activity of Fhit would provide valuable tools for the elucidation of its tumor-suppressive functions. Here we describe the development of a high-throughput screen for the identification of such small-molecule inhibitors of Fhit. Two clusters of inhibitors that decreased the activity of Fhit by at least 90% were identified. Several derivatives were synthesized and exhibited in vitro IC_{50} values in the nanomolar range.

The fragile histidine triad protein (Fhit) is a tumor suppressor, and the associated gene is often mutated in lung,[1] kidney,[2] esophagus,[3] breast,[4] and many other types of human cancer.[5] The gene is located on chromosome 3p14.2, which spans one of the most fragile sites of the genome: FRA3B.[3] Upon complex formation with two molecules of diadenosine triphosphate (Ap_3A), the tumor-suppressive function of Fhit and hence the signaling pathway towards apoptosis are believed to be activated.[5,6] Additionally, Fhit also displays Ap_3Aase activity and cleaves Ap_3A into adenosine monophosphate (AMP) and adenosine diphosphate (ADP). This is believed to terminate Fhit-induced signaling[7] because it was previously reported that Fhit mutants without hydrolase activity still show tumor-suppressive function, whereas Ap_3A-binding mutants are inactive in this respect.[6, 8] Furthermore, reinstatement of the *FHIT* gene in Fhit-negative tumor cells resulted in significant inhibition of cell proliferation and increased apoptosis in vitro[9–11] and in decreased tumorigenicity in vivo.[6]

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Because the lifetime of the Fhit·Ap_3A complex is believed to determine the level of tumor suppression it is suggested that stress conditions in vivo result in reduced Ap_3Aase activity.[12] On investigation of this hypothesis, it was discovered that Fhit might be regulated by Src-kinase-mediated phosphorylation.[12,13] The importance of Fhit and its enzymatic activity for cellular function was further demonstrated by the observation that a nonhydrolyzable Ap_nA analogue triggers Fhit-dependent and caspase-directed apoptosis.[14]

The exact mechanisms of how Fhit activity and the Fhit·Ap_3A-mediated signaling pathway act and are regulated, however, still remain to be discovered. Small-molecule inhibitors of the enzymatic activity of Fhit would provide tools that could assist in the elucidation of its functions in this context. Only a few molecules that are able to inhibit Fhit's enzymatic activity have been reported to date. Among these, suramin[15] and noncleavable Ap_nA analogues,[16–18] as well as transition metal ions such as Cu^{2+} and Zn^{2+},[19] have been presented over the years. Cu^{2+} and Zn^{2+}, however, inhibit Fhit rather unspecifically because they have many other functions and targets within cells. Suramin also inhibits several different classes of enzymes.[20,21] Reported IC_{50} values of $ZnCl_2$ and suramin towards Fhit are in the micromolar rather than the nanomolar range.[22] Overall, small-molecule inhibitors of Fhit would be of considerable interest but had not been described previously.

In the hope of discovering potent small-molecule inhibitors of Fhit, we set out to develop a high-throughput screening assay to identify inhibitors of the enzymatic activity of Fhit from small-molecule compound libraries. Recently we reported the synthesis and application of **1** (Figure 1 A), a doubly labeled Ap_3A probe capable of undergoing FRET, the fluorescence characteristics of which change drastically upon enzymatic cleavage by Fhit.[22] The cleavage of the FRET pair Sulfo-Cy3/Sulfo-Cy5 can readily be followed by measuring the fluorescence intensity at 590 nm, the maximum emission of the donor Sulfo-Cy3. Therefore, this approach is ideally suited as a high-throughput format for the identification of effectors of Fhit's enzymatic activity.

Using the assay shown in Figure 1 B, we screened 15136 compounds from libraries from ChemBioNet, ChemDiv, and Maybridge, available in the screening facility at the University of Konstanz, for inhibition of Fhit's enzymatic activity. For this purpose, Fhit (6 nM) and screening buffer were placed in 384-well plates and incubated with the appropriate compounds (10 μM) at 25 °C for 30 min. FRET probe **1** (500 nM) was added, and fluorescence intensity with $\lambda_{em} = 590$ nm ($\lambda_{ex} = 535$ nm) was measured after $t_0 = 0$ min and $t_1 = 30$ min. The difference in fluorescence intensity relative to a DMSO control containing none of the small-molecule compound was calculated. In total,

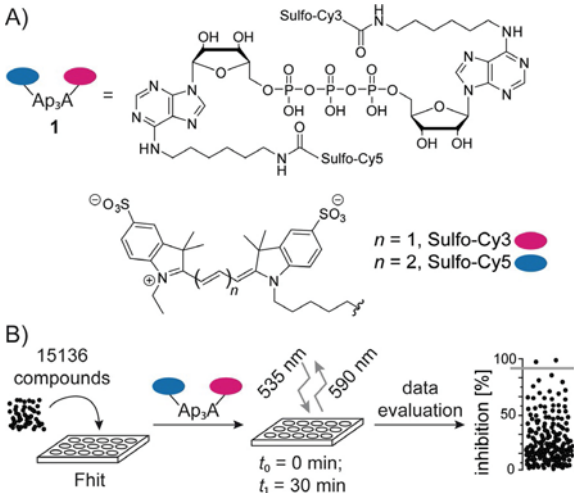


Figure 1. A) Structure of the previously reported doubly fluorescently labeled Ap_3A FRET probe **1**. B) Schematic overview of the screening assay. Potential inhibitors were added to Fhit and incubated for 30 min at 25 °C. Fluorescence intensity with excitation at 535 nm and emission at 590 nm was measured upon addition of FRET-Ap_3A (**1**) at $t_0 = 0$ min and $t_1 = 30$ min.

we found 45 compounds that inhibited the enzymatic activity of Fhit by at least 90% (Figures S1 and S2 in the Supporting Information). The statistical parameters of the high-throughput screening were excellent, with an average *z*-score over all plates of 0.860 ± 0.032 and a strictly standardized mean difference (SSMD) value of -27.244 ± 6.572.

The best initial hit from the screening was compound **2** (Figure 2A), which reduced Fhit activity to less than 2% of that of the DMSO control. To validate this result, compound **2** was synthesized according to a previously reported microwave-assisted procedure from dimedone, 3-amino-5-methylpyrazole, and 4-chlorobenzaldehyde.[23] Compound **2** was then employed at different concentrations in an in vitro dose/response FRET assay, similar to the screening procedure but with measurements taken every 3 min over 60 min (Figure 2B). Corresponding to the linear time response of the cleavage of **1**, a concentration of 1 nM of Fhit was applied in the dose/response assay (Figure S3). In this way, the IC_{50} value of **2** was determined to be (199 ± 10) nM (Figure 2C).

Overall we identified one main structural motif—7,7-dimethyl-4,6,7,8-tetrahydro-1*H*-quinolin-5-one (Scheme 1)—in the screening; it was found in several derivatives among the best 45 hits. Besides the best inhibitor of the screening, compound **2**, with a methylpyrazolo group bridging positions R^1 and R^2, we also discovered inhibitors possessing an indeno motif substituted at R^1 and R^2 (Scheme 1B). The screening results further indicated that bulky groups at R^3 significantly compromise activity, thus indicating that this part of the molecule should be tightly bound to the protein. We therefore chose to improve the inhibitory properties of these initial scaffolds by synthesizing derivatives differing in position R^3.

Hence, we set out to synthesize derivatives (Scheme 1B) based either on the methylpyrazolo motif (cluster I, **2**–**10**) or on the indeno motif (cluster II, **11**–**19**). Lead compound **2** was the only one containing a methylpyrazolo group at R^1 and R^2 in the screening. In the case of the cluster II compounds, *p*-tolyl- (compound **11**), 4-ethylphenyl- (compound **12**), and thiophen-2-yl-substituted (compound **19**) derivatives had already been identified in the screening and inhibited Fhit activity by more than 95% at 10 μM.

The synthesis of the compounds of both clusters was carried out in a one-pot manner with microwave-assisted heating (Schemes 1C and D). In the case of cluster I, the commercially available starting materials dimedone, 3-amino-5-methylpyrazole, and the appropriate aldehydes were mixed in a 1:1:1 ratio and heated to 170 °C in aqueous medium with 1.2 equiv. of NEt_3 in a microwave for 15 min. Precipitation with EtOH/H_2O (1:1) and subsequent filtration yielded the products. The synthesis of compounds **2**–**10** has been reported previously, except for that of compound **4**, which was synthesized in analogy to the literature.[23,24]

Derivatives of cluster II were synthesized from indane-1,3-dione, 3-amino-5,5-dimethylcyclohex-2-enone, and the appropriate aldehydes in a 1:1:1 ratio. *p*-TsOH was added, and the mixtures were heated in a microwave at 150 °C in H_2O for 10 min. The reactions were quenched with 10% NaOH, and the compounds were purified by silica gel column chromatography and reversed-phase medium-pressure column chromatography. The synthesis of compounds **14** and **15** has been described previously.[25,26] To the best of our knowledge, the other cluster II compounds had not been synthesized before. In total, 18 compounds were synthesized. The yields are given in Scheme 1E.

Next, we tested the inhibitory potential of the synthesized compounds by use of the in vitro FRET assay described above. Four compounds—**12**, **13**, **14**, and **15**—were found to inhibit

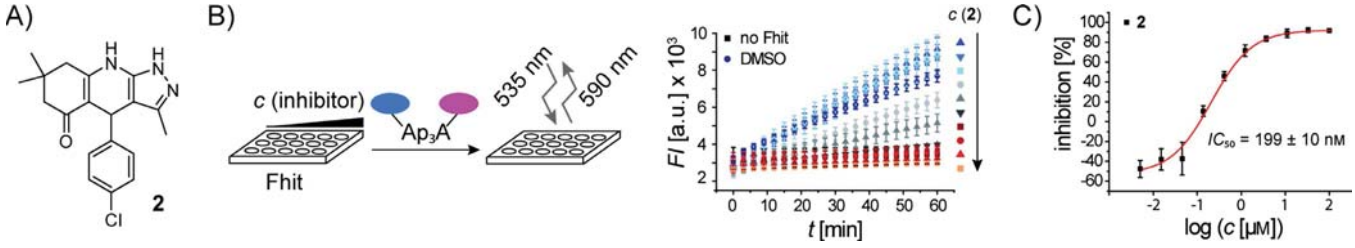
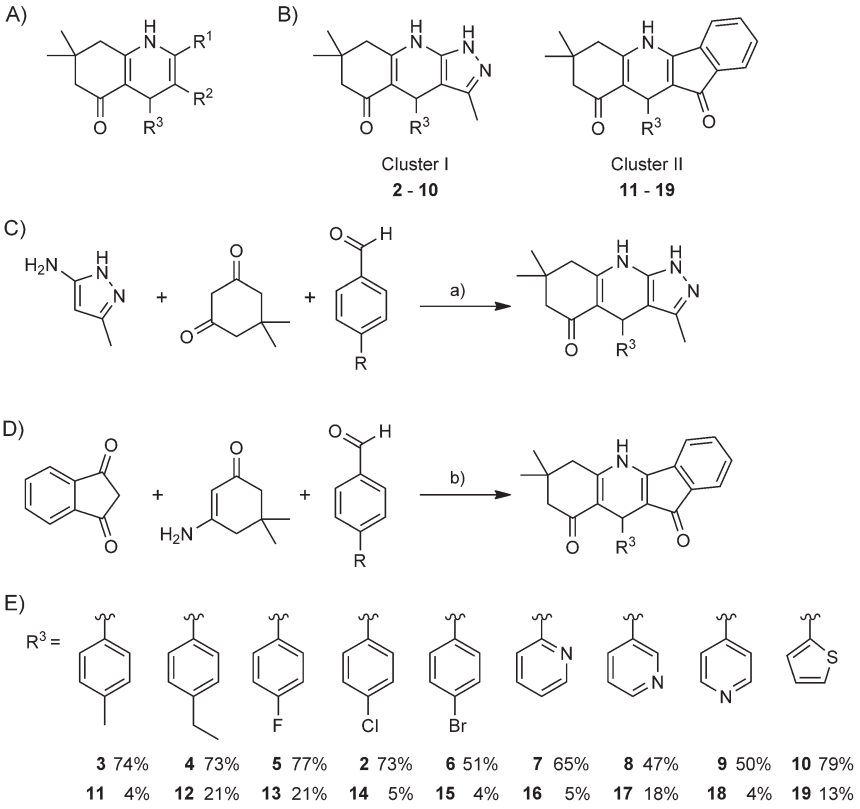


Figure 2. A) Compound **2**, the best initial hit found in the screening assay. B) FRET assay to determine the inhibitory potential of the best compounds found in the screening. Increasing concentrations of small-molecule compounds were incubated with Fhit, after which FRET-Ap_3A was added. Measurements of the fluorescence intensity of the donor emission at 590 nm were made every 3 min over 1 h. The resulting plot of fluorescence intensity (FI) against time shows a decrease in FI when inhibitor is added in increasing concentrations. C) Dose/response curve of **2** against Fhit.



Scheme 1. A) Recurring structural motif found in the identified Fhit inhibitors. B) Structures of the two clusters of Fhit inhibitors found in the screen. Synthesis schemes for C) cluster I, and D) cluster II by a one-pot protocol with microwave irradiation. a) Et_3N, H_2O, MW, 170 °C, 15 min; b) *p*-TsOH, H_2O, MW, 150 °C, 10 min. E) Synthesized derivatives and yields of synthesis.

Fhit more potently than lead compound **2**. Interestingly, all of these compounds are from cluster II, and all the derivatives based on this structural motif are better inhibitors than the corresponding cluster I derivatives with the same R^3 group.

The halogenated and alkylated structures have higher inhibitory potentials than the pyridinyl- and the thiophen-2-yl-substituted derivatives. The Br-substituted compound **15** is the most potent inhibitor, with an IC_{50} value of (49 ± 7) nM, followed by its Cl- [compound **14**, (51 ± 32) nM], Et- [compound **12**, (125 ± 44) nM], and F-substituted [compound **13**, (154 ± 55) nM] counterparts. The effect of the halogenated and alkylated compounds might be due to the altered electronic properties of the aromatic systems. The IC_{50} values of all synthesized compounds are shown in Table 1 (the associated dose/response curves are displayed in Figure S4).

Because the synthesized compounds are conjugated π-systems, an additional negative control was performed, studying the influence of the potential inhibitors on the FRET assay being studied when no Fhit was applied. We found that all inhibitors had no effect on the fluorescent read-out (Figure S5). Furthermore, as shown by the results of the structure–activity relationship, slight changes in compound structure that do not alter the π-system can alter the inhibitory potential significantly (e.g., see IC_{50} values of compounds **2** and **9**). Furthermore, several other compounds with conjugated π-systems were screened and did not show any inhibition of Fhit (Figure S6). This shows that the depicted system is highly sensitive to slight structural changes in the small-molecule system, excluding the possibility that just any extended aromatic system is an inhibitor.

Table 1. IC_{50} values of all synthesized derivatives.

Cluster I compounds	IC_{50} [nM]	Cluster II compounds	IC_{50} [nM]
3	589 ± 160	**11**	275 ± 1
4	388 ± 26	**12**	125 ± 44
5	762 ± 105	**13**	154 ± 55
2	199 ± 10	**14**	51 ± 32
6	379 ± 54	**15**	49 ± 7
7	6682 ± 1948	**16**	4245 ± 459
8	2225 ± 721	**17**	1654 ± 241
9	16840 ± 3160	**18**	5204 ± 261
10	925 ± 198	**19**	499 ± 187

Compound **15** was further tested on its effect on Fhit when the natural substrate Ap_3A was used instead of probe **1**. As analyzed by HPLC, Fhit was competitively inhibited by **15** when the Fhit/inhibitor ratio was 1:1000 and higher, a value that corresponds well with that determined in the FRET assay (Figure S7 A).

To examine details about the enzymatic inhibition of Fhit further, another FRET-based assay was conducted. Here increasing amounts of substrate **1** were applied to preincubated mixtures of inhibitor **15** and Fhit. A higher turnover was achieved with rising concentrations of **1** (Figure S7 B). This sug-

gests that **15** does not covalently modify enzyme residues that are crucial for catalysis.

Next, all compounds were tested for their cytotoxic properties in the HEK 293T and lung cancer H1299 cell lines that are often used in Fhit research.[14,27–29] A colorimetric assay that determines the metabolic activity of cells, and thus cell viability, as a function of the reduction of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into insoluble formazan by NAD(P)H-dependent oxidoreductases was employed.[30] The results for the halogenated cluster II compounds **13**, **14**, and **15** are displayed in Figure 3 A and B (the MTT results for all compounds are displayed in Figure S8). All three compounds show similar cytotoxicity. Interestingly, HEK 293T cells are more sensitive to the Fhit inhibitors than

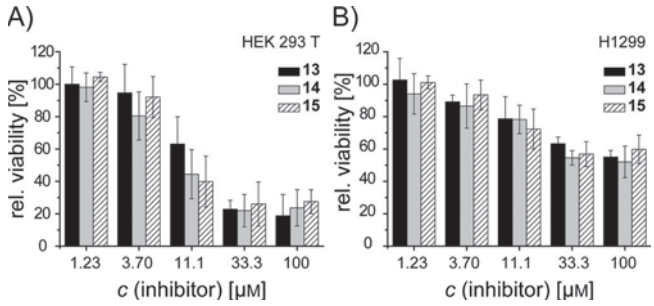


Figure 3. MTT assay of halogenated compounds of cluster II derivatives **13**, **14**, and **15** in A) HEK 293T, and B) H1299 cells. Concentrations lower than those displayed did not alter the relative cell viability.

H1299 cells. The viability of the former is already affected in the 10 μM range, whereas the latter still exhibit more than 50% viability even when 100 μM of compound are applied. Overall, the compounds show moderate to no cytotoxicity, depending on the cell line, and are therefore ideally suited for further in vivo studies.

In conclusion, we have developed a high-throughput assay using FRET-Ap_3A probe **1** to identify a main structural motif as a potential small-molecule inhibitor of the activity of the tumor suppressor Fhit. On this basis, we synthesized 18 compounds, the synthesis of several of them not previously reported, and identified five of these—**2**, **12**, **13**, **14**, and **15**—that inhibited Fhit with IC_{50} values of <200 nM. The best inhibitor, compound **15**, had an IC_{50} value of (49 ± 7) nM and was bound by Fhit in a reversible manner.

The compounds were further tested for their cytotoxic properties towards HEK 293T and H1299 cells, two cell lines commonly studied in Fhit research. The inhibitors showed moderate to no cytotoxicity, with HEK 293T cells seeming to be more affected than H1299 cells. Interestingly, HEK 293T cells constitutively express Fhit,[27] whereas H1299 are Fhit-negative.[31]

Overall we identified several highly effective Fhit inhibitors that should be useful for study of the enzymatic activity of Fhit in more detail under various conditions. However, in human cells seven members of the histidine triad (HIT) protein family, including Fhit, have been described.[32,33] Because the applied high-throughput screening was not designed to discriminate between the members, inhibition of other HIT family proteins cannot be excluded. Nevertheless, the discovered molecular scaffolds could eventually contribute to the development of new compounds for use in anticancer therapies to trigger Fhit-dependent apoptosis.

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Keywords: Ap_3A · enzyme inhibitors · Fhit · FRET · nucleotides

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