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Small molecule inhibitors of the tumor suppressor Fhit

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Abstract: 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) which is believed to terminate Fhit-mediated signaling. How the catalytic activity of Fhit is regulated and how the Fhit-Ap_3A complex exerts 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 were identified that decreased activity of Fhit by at least 90%. Several derivatives were synthesized and exhibited IC_{50} values *in vitro* in the nM range.

The fragile histidine triad protein (Fhit) is a tumor suppressor and the respective 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 activated.[5, 6] Besides, Fhit also comprises Ap_3Aase activity and cleaves Ap_3A into adenosine monophosphate (AMP) and adenosine diphosphate (ADP). This is believed to terminate Fhit-induced signaling[7] since 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] Because the lifetime of the Fhit-Ap_3A complex is determining the level of tumor suppression it is suggested that stress conditions *in vivo* result in reduced Ap_3Aase activity.[12] Following 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 has been further demonstrated by the observation that a non-hydrolyzable 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 are acting and 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 have been reported to date that are able to inhibit Fhit's enzymatic activity. Among these, Suramin[15] and non-cleavable Ap_nA analogues[16–18] have been presented over the years next to transition metal ions like Cu^{2+} and Zn^{2+}.[19] The latter, however, inhibit Fhit rather unspecific as 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 are of considerable interest and not described so far.

To discover potent small-molecule inhibitors of Fhit, we set out to develop a high-throughput screening assay to identify inhibitors against the enzymatic activity of Fhit from small molecule libraries. Recently, we reported the synthesis and application of a doubly labeled Ap_3A probe **1** (**Figure 1A**), capable of undergoing Förster Resonance Energy Transfer (FRET), whose fluorescence characteristics change drastically upon enzymatic cleavage by Fhit.[22] The cleavage of the FRET pair Sulfo-Cy3/Sulfo-Cy5 can be readily 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.

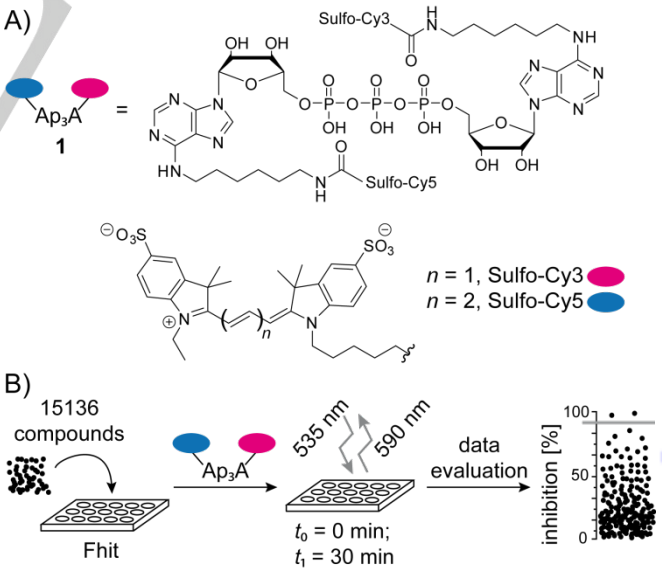

Figure 1. A) Structure of the previously reported doubly fluorescently labeled Ap_3A FRET probe **1**. B) Schematic overview of the screening assay. Potential inhibitors were added to Fhit and incubated for 30 min at 25 °C. Fluorescence intensity with excitation at 535 nm and emission at 590 nm was measured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

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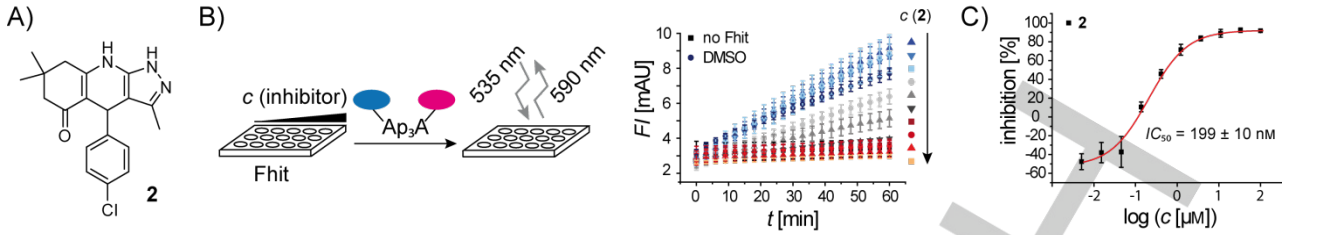


Figure 2. A) The best initial hit found in the screening assay (**2**). B) FRET assay to determine the inhibitory potential of the best compounds found in the screening. Increasing concentrations of small molecules were incubated with Fhit before FRET-Ap$_3$A was added. Time points were taken every 3 min over 1 h and the fluorescence intensity of the donor emission was measured at 590 nm. The resulting plot of fluorescence intensity (FI) against time shows a decrease of FI when inhibitor is added in increasing concentrations. (C) Dose response curve of **2** against Fhit.

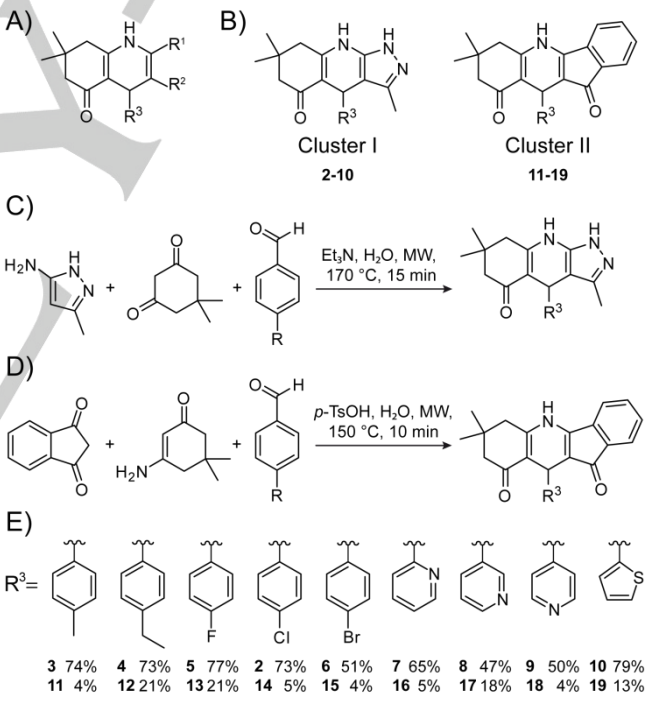
Using the assay shown in **Figure 1B**, we screened 15.136 compounds for inhibition of Fhit's enzymatic activity from libraries from ChemBioNet, ChemDiv and Maybridge that were available in the screening facility at the University of Konstanz. For this purpose, Fhit (6 nM) and screening buffer were placed in 384-well plates and incubated with the respective compounds (10 μM) at 25 °C for 30 min. FRET-probe **1** (500 nm) was added and fluorescence intensity with emission at 590 nm (535 nm excitation) was measured after t_0 = 0 min and t_1 = 30 min. The difference in fluorescence intensity was then calculated relative to a DMSO control in the absence of any small molecule. In total, we found 45 compounds that inhibited the enzymatic activity of Fhit by at least 90% (**Figure S1**, **S2**). 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 SSMD value of −27.244 ± 6.572.

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

Overall we identified one main structural motive, dimethyl-hexahydro-quinolinone (**Scheme 1**), in the screening that was found with several derivatives among the best 45 hits. Besides the best inhibitor of the screening (**2**) with a methyl-pyrazolo group bridging positions R^1 and R^2, we discovered inhibitors that have an indeno residue substituted at R^1 and R^2 (**Scheme 1B**). The screening results further indicated that bulky groups at R^3 significantly compromise activity 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 of the methyl-pyrazolo-motive (cluster I, **2**–**10**) and the indeno motive (cluster II, **11**–**19**) (**Scheme 1B**). Lead compound **2** was the only one containing a methyl-pyrazolo group at R^1 and R^2 in the screening. In case of the cluster II compounds, toluyl (**11**), 4-ethylphenyl (**12**) and 2-thiophenyl-substituted (**19**) derivatives were already identified in the screening and inhibited Fhit activity by more than 95% at 10 μM.

The synthesis of both clusters was carried out in a one-pot manner with microwave-assisted heating (see **Schemes 1C** and **3D**). In case of cluster I, the commercially available starting materials dimedone, 3-amino-5-methyl-pyrazole and the respective aldehydes were mixed in a 1:1:1 ratio and were heated in aqueous medium with 1.2 eq. of NEt_3 to 170 °C 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** was previously reported, except for compound **4** that was synthesized in analogy to the literature.[23, 24]



Scheme 1. A) Structural motive found recurring in the identified Fhit inhibitors. B) Structure of the two clusters of Fhit inhibitors found in the screen. C), D) Synthesis scheme for cluster I (C) and cluster II (D) using a one-pot protocol with microwave irradiation. E) Synthesized derivatives and yield of synthesis.

Derivatives of cluster II were synthesized from 1,3-indanedione, 5,5-dimethyl-3-amino-cyclohex-2-enone and the respective aldehydes in a 1:1:1 ratio. *p*-TsOH was added and the mixture was reacted in a microwave at 150 °C for 10 min in H_2O. The reaction was 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** was described previously.[25, 26] To the best of our knowledge, the other cluster II compounds have not been synthesized before. In total, 18 compounds were synthesized. The respective yields are depicted in **Scheme 1E**.

Next, we tested the inhibitory potential of the synthesized compounds using the *in vitro* FRET assay described above. Four compounds, **12**, **13**, **14** and **15**, were found to inhibit Fhit more potently than lead compound **2**. Interestingly, all of these compounds derive from cluster II and all the derivatives of this structural motive are better inhibitors than the respective derivatives of cluster I with the same R^3 group.

The halogenated and alkylated structures have a higher inhibitory potential than the pyridinyl- and thiophenyl-substituted derivatives. The Br-substituted compound **15** is the most potent inhibitor with an IC_{50} value of 49 ± 7 nM, followed by Cl (**14**, 51 ± 32 nM), Et (**12**, 125 ± 44 nM) and F (**13**, 154 ± 55 nM). 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 respective dose-response curves are displayed in **Figure S4**).

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

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

Compounds Cluster I	IC_{50} [nM]	Compound Cluster II	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	6,682 ± 1,948	**16**	4,245 ± 459
8	2,225 ± 721	**17**	1,654 ± 241
9	16,840 ± 3,160	**18**	5,204 ± 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 to the conducted FRET assay (**Figure S7A**).

To further examine details about the enzymatic inhibition of Fhit, another FRET-based assay was conducted in which increasing amounts of substrate **1** were applied to pre-incubated mixtures of inhibitor **15** and Fhit. A higher turnover was achieved with rising concentrations of **1** (**Figure S7B)**. This suggests that **15** acts as a competitive inhibitor and that the substrate is able to compete with the binding of **15** and displace it.

Next, all compounds were tested for their cytotoxic properties in HEK 293T and lung cancer H1299 cell lines, that are often used in Fhit research.[14, 27–29] Therefore a colorimetric assay was employed which 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-diphenyltetra-zolium bromide (MTT) into insoluble formazan by NAD(P)H-dependent oxidoreductases.[30] The results for the halogenated cluster II compounds **13**, **14** and **15** are displayed in **Figure 3A** and **3B** (the MTT results for all compounds are displayed in **Figure S8**). All three compounds show similar cytotoxicity. Interestingly, HEK 293T cells are more sensitive towards the Fhit inhibitors than H1299 cells. The viability of the former is already affected in the 10 µM range, while the latter still exhibit more than 50% viability even when 100 µM of compound are applied. Overall, the compounds show a moderate to no cytotoxicity, depending on the cell line and are therefore ideally suited for further *in vivo* studies.

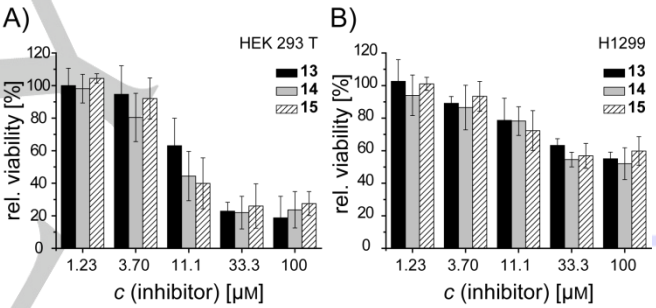


Figure 3. MTT assay of halogenated compounds of cluster II derivatives (**13**, **14**, **15**) in HEK 293T (A) and H1299 (B) cells. Lower concentrations than the ones displayed did not alter the relative cell viability.

In conclusion, we developed a high-throughput assay using FRET-Ap_3A probe **1** to identify a main structural motive as potential small molecule inhibitor of the activity of the tumor suppressor Fhit. Based on this, we synthesized 18 compounds, the synthesis of several thereof previously unknown, and identified five of these, **2**, **12**, **13**, **14** and **15**, that inhibited Fhit with IC_{50} values of < 200 nM. The best inhibitor (**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 in HEK 293T and H1299 cells, two commonly studied cell lines in Fhit research. The inhibitors showed moderate to no cytotoxicity, whereby HEK 293T cells seem to be more affected than H1299. Interestingly, HEK 293T cells constitutively express Fhit,[27] while H1299 are Fhit negative.[31]

Overall we identified several highly effective Fhit inhibitors that will be useful to study the enzymatic activity of Fhit in more detail under various conditions. However, in human cells seven members of the histidine triad (HIT) protein-family are described,

including Fhit.[32, 33] Since 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 may eventually contribute to the development of novel compounds that can be used in anti-cancer therapies to trigger Fhit-dependent apoptosis.

Experimental Section

Experimental details are given in the Supporting Information.

Acknowledgements

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Keywords: Fhit • Inhibitors • Ap_3A • FRET • Nucleotides

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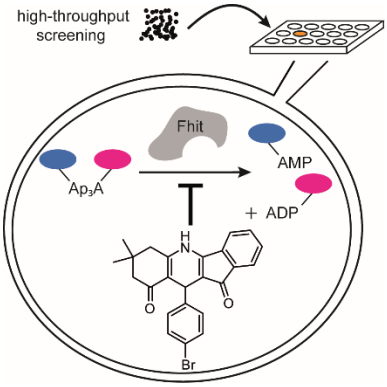
COMMUNICATION

Small molecule inhibitors have been identified and developed by high-throughput screening and organic synthesis that suppress enzymatic hydrolysis of diadenosine triphosphate (Ap_3A) into adenosine monophosphate (AMP) and adenosine diphosphate (ADP) by the human tumor suppressor Fhit.



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