Signaling Molecules

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Selective Monitoring of the Enzymatic Activity of the Tumor Suppressor Fhit**

*Stephan M. Hacker, Franziska Mortensen, Martin Scheffner, and Andreas Marx**

Abstract: *Cancer is a leading cause of death worldwide. Functional inactivation of tumor suppressor proteins, mainly by mutations in the corresponding genes, is a key event in cancer development. The fragile histidine triade protein (Fhit) is a tumor suppressor that is frequently affected in different cancer types. Fhit possesses diadenosine triphosphate hydrolase activity, but although reduction of its enzymatic activity appears to be important for exerting its tumor suppressor function, the regulation of Fhit activity is poorly understood. Here, we introduce a novel fluorogenic probe that is suited to selectively analyze the enzymatic activity of Fhit in extracts derived from human cells. This novel method will allow in-depth insight into the mechanisms involved in Fhit regulation in biologically relevant setups and, thus, into its role in the development of cancer.*

Cancer is currently one of the most frequent causes of death worldwide.[1] During cancer development ("carcinogenesis"), mutations in cell regulatory genes accumulate which in a simplified view provide cells with carcinogenic properties including immortality, uncontrolled cell proliferation, and tissue invasion.[2] Inactivation of so-called tumor suppressor proteins that have negative growth-regulatory functions plays a key role during this process.[3] The fragile histidine triade protein (Fhit), encoded by the fragile FRA3B locus, is one of these tumor suppressors[4] and is frequently dysregulated at early stages of the development of different types of cancer;[5] however, how this dysregulation contributes to cancer development remains enigmatic.

Fhit forms homodimers and has the activity of a diadenosine triphosphate hydrolase (Ap_3Aase), cleaving diadenosine triphosphate (Ap_3A) to adenosine monophosphate (AMP) and adenosine diphosphate (ADP, Figure 1a).[7] Experimental evidence indicates that the hydrolytic activity of Fhit is not needed for its function as a tumor suppressor,[4] whereas the binding of Ap_3A is crucial.[8] In response to cellular stress, levels of Ap_3A increase, inducing activation of the tumor suppressor function of Fhit by complex formation.[5] However, due to the intrinsic Ap_3Aase activity of Fhit, this tumor-suppressive complex would have a very short lifetime. Thus, it is assumed that upon stress the hydrolytic activity of Fhit is down-modulated.[9] Indeed, overexpression experiments in cell culture and experiments with recombinant Fhit suggest that the Ap_3Aase activity of Fhit may be regulated in part by Src kinase mediated phosphorylation.[9,10] However, the actual network that regulates Fhit in cells remains to be elucidated. As Ap_3A levels in human cells are a measure for the presence or absence of Fhit,[11] fluorogenic Ap_3A analogues would be valuable tools to study its enzymatic activity in complex biological environments such as cell extracts.

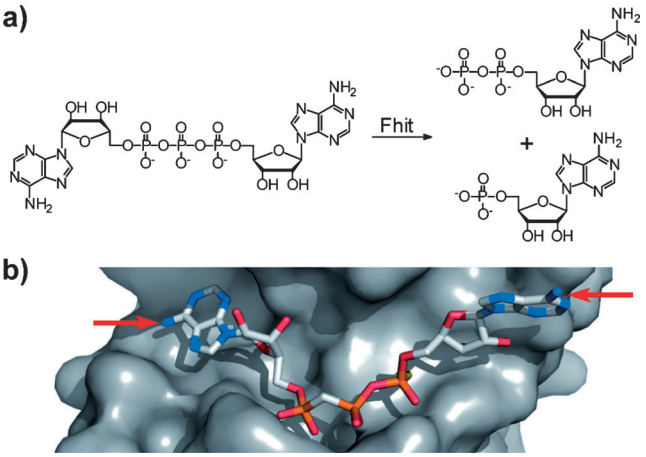

Figure 1. a) Enzymatic activity of Fhit cleaving Ap_3A to ADP and AMP. b) Enlarged view of the crystal structure of Fhit (PDB 1FHI, Connolly surface) bound to a non-hydrolyzable Ap_3A analogue (rods).[6] Red arrows indicate the two N6-positions of Ap_3A that were modified in this study.

Fluorogenic substrates of Fhit that have been reported so far comprise an Ap_3A-based probe containing fluorescent adenine analogues that fluoresce at short wavelengths which is disadvantageous for many applications.[12] Furthermore, a GTP analogue was used;[13] however, it is questionable whether GTP analogues in general are suited to selectively sense Ap_3Aase activity. In any case, none of the reported approaches can be used to study the enzymatic activity of endogenous Fhit, for example in extracts derived from cells or cell lines.

We therefore set out to develop an Ap_3A analogue modified with two synthetic dyes able to undergo Förster resonance energy transfer (FRET) as a fluorogenic substrate

[*] Dr. S. M. Hacker, Dipl.-Nat. F. Mortensen, Prof. Dr. M. Scheffner, Prof. Dr. A. Marx
Department of Chemistry and Department of Biology
Konstanz Research School Chemical Biology, University of Konstanz
Universitätsstrasse 10, 78457 Konstanz (Germany)
E-mail: andreas.marx@uni-konstanz.de

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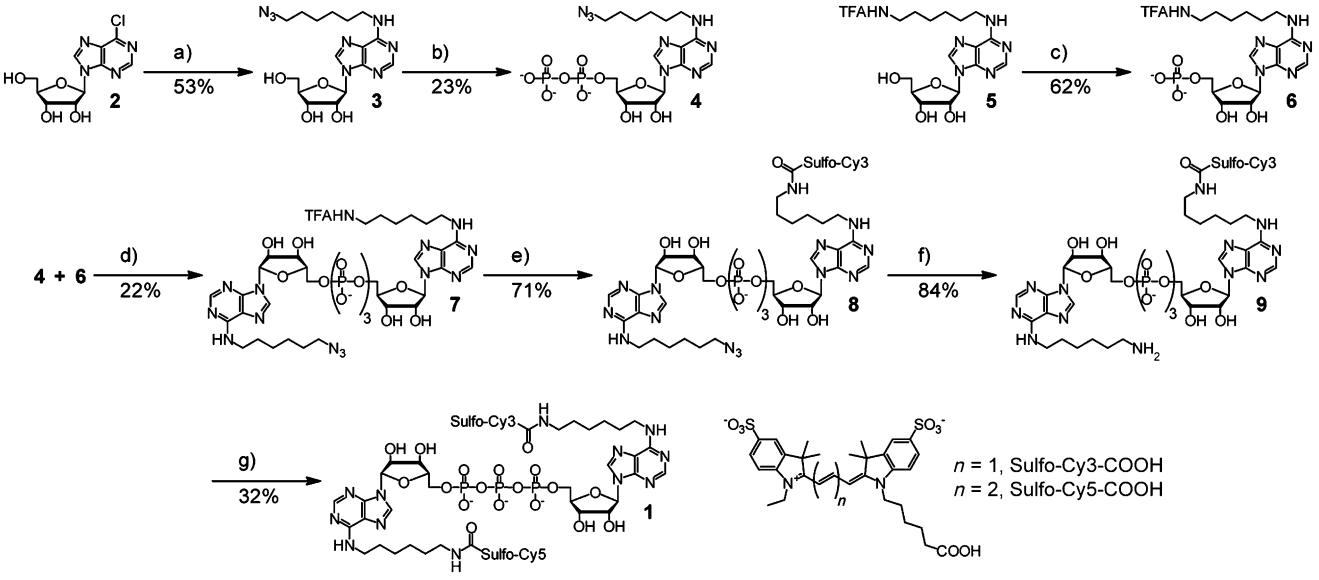
for Fhit. This analogue should be cleaved with high efficiency exclusively by reaction with Fhit and show large changes in fluorescence intensity upon cleavage at wavelengths suitable for studies in complex biological systems. By inspection of the crystal structure of Fhit bound to a nonhydrolyzable Ap_3A analogue (Figure 1b),[6] we found that both N6-positions of Ap_3A are largely accessible and should thus be modifiable without significantly perturbing acceptance by Fhit. We therefore designed probe **1** (Scheme 1) as a fluorogenic tool to study the enzymatic activity of Fhit. As dyes, we utilized Sulfo-Cy3 and Sulfo-Cy5 as they have already been successfully applied to study nucleotide cleavage.[14]

The synthesis of probe **1** started from 6-chloro-9-(β-D-ribofuranosyl)purine[15] **2** (Scheme 1), which was treated with 6-azidohexylamine[16] resulting in compound **3** in 53% yield. Treatment with phosphorus oxychloride and subsequent reaction with bis(tributylammonium) phosphate gave compound **4** in 23% yield. To synthesize the orthogonally protected second N6-modified adenosine, compound **5**[17] was phosphorylated at the O5′-position to give **6** in 62% yield. The two nucleotides were coupled in analogy to a published procedure[18] to give the Ap_3A analogue **7** in 22% yield. Attachment of the first dye was achieved by deprotection of the trifluoroacetamide with sodium hydroxide, followed by coupling of the Sulfo-Cy3-*N*-hydroxysuccinimide (NHS) ester to give compound **8** in 71% yield. The azide was reduced by Staudinger reduction to provide compound **9** in 84% yield. The synthesis was completed by coupling of the Sulfo-Cy5-NHS ester to result in probe **1** in 32% yield.

Next, we tested whether probe **1** can serve as a fluorogenic substrate for Fhit. Whereas probe **1** is stable in the absence of the enzyme, recombinant Fhit quantitatively cleaves probe **1**, as detected by RP-HPLC (Figure S1 in the Supporting Information). The fluorescence spectra of the cleaved and noncleaved state of probe **1** (Figure S2 in the Supporting Information) show an 86-fold difference in the ratio of the fluorescence intensity of the donor and the acceptor. After quantitative cleavage only minimal fluorescence of the acceptor Sulfo-Cy5 was detected due to direct excitation of this dye. Subsequently, we studied the time-course of the enzymatic reaction of Fhit using probe **1** (Figure 2a). The fluorescence spectra of probe **1** in the presence of Fhit show a decrease of the acceptor fluorescence accompanied by an increase of the donor fluorescence over time. This was not observed in the absence of Fhit (Figure S3, Supporting Information). Using the donor fluorescence intensity as a measure for the cleavage of probe **1**, the turnover rate of probe **1** by Fhit can be calculated (Figure 2b and Figure S4 in the Supporting Information). The rate depends linearly on the concentration of wild-type Fhit over at least two orders of magnitude and enzymatic activity was detected at concentrations of Fhit in the sub-nanomolar range. No enzymatic activity was detected over the whole range of protein concentrations for the catalytically inactive Fhit H96N mutant.[7] Performing the experiment with wild-type Fhit at varying concentrations of probe **1** (Figure S5 in the Supporting Information) revealed $K_M = (2.39 \pm 0.08)$ μM, which is on the same order of magnitude as that reported for the natural substrate,[10] demonstrating the good acceptance of probe **1** by Fhit. To further explore the applicability of probe **1** to characterize the enzymatic activity of Fhit, we tested two known inhibitors of Fhit (Figure S6 in the Supporting Information).[12] Suramin and zinc chloride inhibit Fhit with IC_{50} values of (1.10 ± 0.17) μM and (43.7 ± 9.2) μM, respectively. This is in good agreement with the reported IC_{50} values



Scheme 1. Synthesis of probe **1**. Conditions: a) 6-azidohexylamine, ethanol, 3 h, reflux; b) 1. $POCl_3$, trimethylphosphate, 1 h, 0°C; 2. $(Bu_3NH^+)_2HPO_4^-$, Bu_3N, DMF, 30 min, RT; 3. 0.1 M TEAB, 30 min, RT; c) 1. $POCl_3$, trimethylphosphate, 1 h, 0°C; 2. 0.1 M TEAB, 30 min, RT; d) 1. **6**, Et_3N, TFAA, AcCN, 10 min, RT; 2. *N*-methylimidazole, Et_3N, 10 min, 0°C; 3. **4**, 4 Å molecular sieves, DMF, 2 h, RT; 4. 0.1 M TEAB, 30 min, RT; e) 1. 0.1 M NaOH, 3 h, RT; 2. Sulfo-Cy3-NHS ester, 0.1 M $NaHCO_3$, DMF, 12 h, RT; f) TCEP-HCl, water, methanol, Et_3N, 12 h, RT; g) Sulfo-Cy5-NHS ester, 0.1 M $NaHCO_3$, DMF, 12 h, RT.

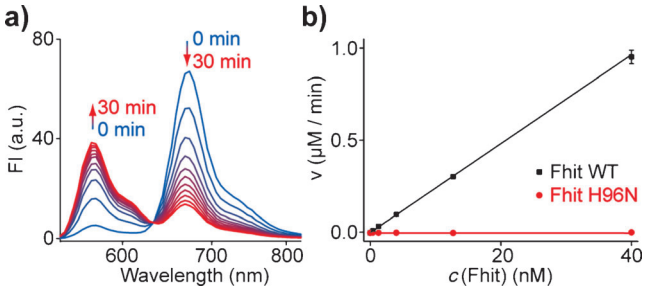


Figure 2. a) Change of the fluorescence spectra of probe **1** excited at 490 nm over time in the presence of Fhit. FI: fluorescence intensity; a.u.: arbitrary units. b) Dependence of the rate (v) of probe **1** turnover on the concentration of recombinant wild-type Fhit (Fhit WT) and the Fhit H96N mutant. Data represent mean ± standard error of triplicates. Experimental details are given in the Supporting Information.

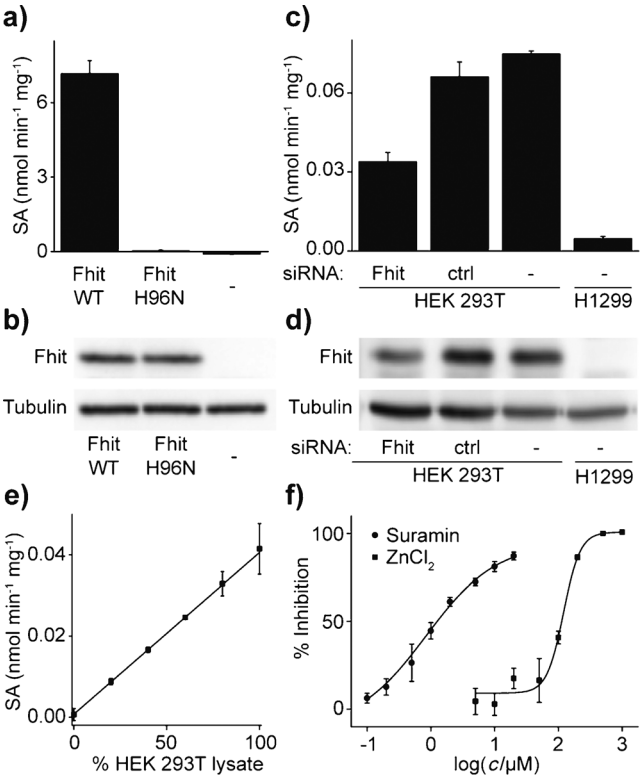


Figure 3. a) Specific activity (SA) of probe **1** turnover in cell lysates of Fhit-negative H1299 cells[19] that were transfected with expression constructs for wild-type Fhit (Fhit WT), the catalytically inactive Fhit H96N mutant, and an empty vector. Fhit variants were expressed as HA-tagged constructs. b) Western blot of the cell lysates applied in (a) using tubulin as a loading control and α-HA antibody for the detection of the HA-Fhit variants. c) Specific activity (SA) of probe **1** turnover in lysates of Fhit-positive HEK 293T cells[20] treated with siRNA against Fhit, control siRNA, and without transfection as compared to Fhit-negative H1299 cells. d) Western blot of the cell lysates applied in (c) using tubulin as a loading control and α-Fhit antibody for the detection of endogenous Fhit. e) Specific activity (SA) of probe **1** turnover in mixtures of cell lysates of Fhit-positive HEK 293T cells and Fhit-negative H1299 cells at constant total protein concentration. f) Concentration-dependent inhibition of enzymatic activity of Fhit by two known inhibitors. All data represent mean ± standard error of triplicates. Experimental details are given in the Supporting Information.

in the unmodified system.[12] Furthermore, probe **1** is not cleaved by the Lewis acid zinc chloride alone.

Next, we set out to explore whether the enzymatic activity of Fhit can also be detected in lysates derived from human cells. For this purpose, we first used the H1299 lung cancer cell line that is known to be Fhit deficient (Figure 3a,b and Figure S7 in the Supporting Information).[19] We transfected these cells with expression constructs for wild-type Fhit, the catalytically inactive Fhit H96N mutant, and a vector without insert (empty vector) as a control. 24 h after transfection, whole cell extracts were prepared and Fhit activity was determined. The generation of a fluorescent signal was detected exclusively upon incubation of **1** in the presence of the extract containing wild-type Fhit. No significant signal generation was observed in the other cases, indicating that probe **1** is a selective substrate for Fhit and is stable in cell extracts (i.e. it does not generate a background signal).

To characterize the functions of Fhit in a biologically relevant setting, it would be important to be able to study endogenous Fhit. Hence, we utilized HEK 293T human embryonic kidney cells that endogenously express Fhit (Figure 3c,d and Figure S8 in the Supporting Information).[20] We found that probe **1** is processed in HEK 293T extracts, whereas it is stable in H1299 cell lysate that does not contain Fhit. This demonstrates that Ap_3A is indeed mainly converted by Fhit in human cells[11] and that this specificity is maintained with probe **1**. To further prove that this activity is caused by endogenous Fhit, we performed an siRNA experiment. The cell lysate of HEK 293T cells treated with siRNA targeted against Fhit mRNA showed a roughly twofold decrease in enzymatic Fhit activity as compared to that of the cells treated with control siRNA or untreated cells. As this correlates with the decrease observed for Fhit protein level (Figure 3d), we can conclude that the cleavage of probe **1** in HEK 293T cell extracts is caused by the endogenous Fhit protein.

To further demonstrate that the assay is suitable for studying effectors of the enzymatic activity of endogenous Fhit, we then checked for the linearity of the assay towards endogenous Fhit protein levels (Figure 3e and Figure S9 in the Supporting Information). For this purpose, we mixed different amounts of Fhit-positive HEK 293T cell lysate with Fhit-negative H1299 cell lysate to ensure a constant total protein amount in the assay. Indeed, the amount of probe **1** cleaved depends linearly on the fraction of HEK 293T lysate used, and thus also in cell lysates, Ap_3Aase activity linearly depends on the amount of Fhit protein present.

The results obtained so far indicate that probe **1** should be suitable for studying the effects of Fhit inhibitors in HEK 293T cell extracts in a quantitative manner (Figure 3f and Figure S10 in the Supporting Information). Suramin[12] inhibits endogenous Fhit in cell extracts with an IC_{50} of (0.84 ± 0.22) μM, which is comparable to the value observed with recombinant enzyme. Zinc chloride[12] also inhibits endogenous Fhit in cell lysates albeit with an increased IC_{50} of (121 ± 5) μM, which may be explained by the presence of other zinc-binding components in cell lysates resulting in a lower concentration of free zinc ions.

In conclusion, we have presented a novel approach to study the enzymatic activity of the tumor suppressor Fhit. For this purpose we developed an Ap_3A analogue modified with two fluorophores able to undergo FRET; this analogue is similarly well accepted by the Fhit protein as the natural substrate. The large change of its fluorescence characteristics upon cleavage facilitates the robust and selective detection of the enzymatic activity of Fhit, even at physiologically relevant levels of Fhit (i.e. in cell extracts derived from human cell lines). The presented method will therefore be an invaluable tool to analyze the regulation of Fhit under physiological conditions and in this way to obtain insight into its role in the early stages of cancer development. Furthermore, it will be applicable to identifying and characterizing positive as well as negative effectors of the enzymatic activity of Fhit.

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