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Development and Biological Evaluation of Acyl Protein Thioesterase 1 (APT1) Inhibitors**

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Lipidation of proteins is often an unalterable prerequisite for correct biological function. Prime examples are the N and H isoforms of the signal transducing Ras protein, which in the normal and oncogenic state are anchored to the plasma membrane by means of *S*-farnesylation and *S*-palmitoylation at their C terminus and which have to be palmitoylated to exert their full biological activity.[1,2] Inhibition of the enzyme protein farnesyltransferase has opened up an unprecedented opportunity for the treatment of tumors carrying a mutation in the Ras oncogene.[3] However, the enzyme responsible for palmitoylation of H- and N-Ras and other proteins crucial to biological signaling, like heterotrimeric G proteins, G-protein-coupled receptors, and nonreceptor tyrosine kinases, has not been identified so far. Clearly the development of potent inhibitors for this biocatalyst might open up new opportuni-

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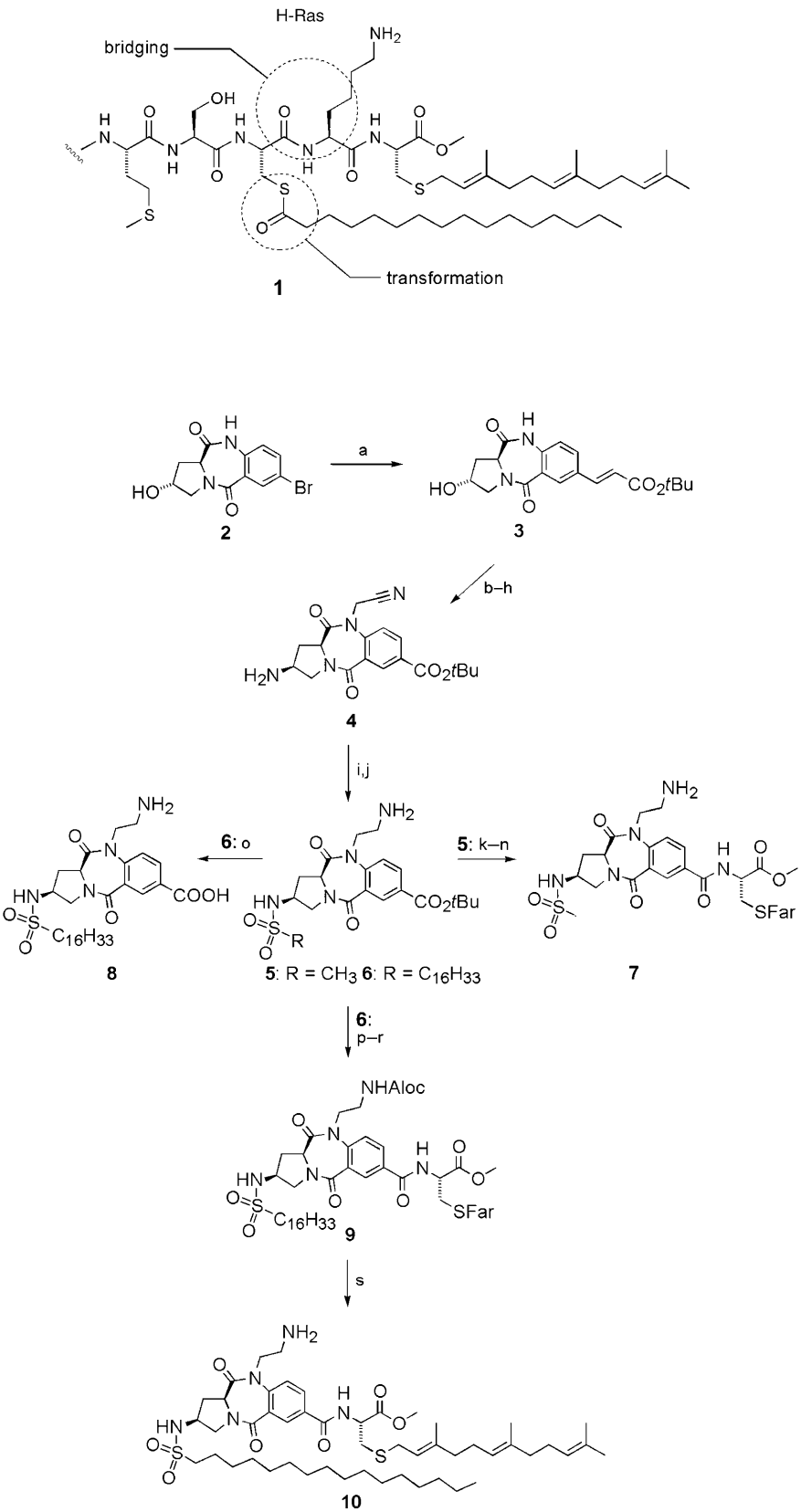
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

ties for the treatment of cancers with a mutation in the H- and N-Ras oncogenes.

In yeast, the Erf2 and Erf4 proteins were found to palmitoylate the yeast Ras oncogene homologues.[4,5] While orthologues of the Erf2 gene can be found in the genomes of various eukaryotes including *Homo sapiens*, proteins with the corresponding *S*-palmitoylating activity have not been identified to date.[6] Erf4 homologues have only been identified in closely related yeasts. Instead, acyl protein thioesterase 1 (APT1) was described as "the first bona fide player" in the regulated thioacylation of intracellular proteins.[7] APT1 was found to depalmitoylate the H-Ras protein and the α subunits of heterotrimeric G proteins. Herein we report a chemical biology approach aimed at determination of the involvement of APT1 in H-Ras palmitoylation in vitro and possibly in vivo.

For a reverse chemical genetics approach,[8] inhibitors to antagonize the biological function of the enzyme were designed based on the structure of the H-Ras C terminus **1** (Scheme 1). To this end, a peptide-imitating benzodia-



Scheme 1. Structure of the lipidated C terminus **1** of the H-Ras protein and synthesis of the corresponding benzodiazepinediones **5**–**10**. a) *tert*-Butyl acrylate, $Pd(OAc)_2$, $P(oTol)_3$, NEt_3, CH_3CN, 100 °C, sealed tube, 97%; b) MsCl, pyridine, 0 °C→RT; c) NaN_3, DMF, 45 °C, 89% over two steps; d) NaH, THF, −40 °C; e) $BrCH_2CN$, −40 °C→RT, 95% over two steps; f) $RuCl_3$, $NaIO_4$, $H_2O/CH_3CN/CCl_4$ (2:1:1); g) Me_3CBr, K_2CO_3, $Et_3(PhCH_2)NCl$, DMA, 55 °C, 90% over two steps; h) H_2, $Pd/BaSO_4$, MeOH, $CHCl_3$, quantitative; i) MsCl, $NEtiPr_2$, DMF, 0 °C→RT, 92%; or $C_{16}H_{33}SO_2Cl$, $NEtiPr_2$, DMF, 0 °C→RT, 82%; j) H_2, $PtO_2{\cdot}H_2O$, EtOH, $CHCl_3$, 85% (**5**); or H_2, $PtO_2{\cdot}H_2O$, EtOH, $CHCl_3$, 81% (**6**); k) AlocCl, NEt_3, CH_2Cl_2, 83%; l) TFA/CH_2Cl_2 (1:1); m) H-Cys(Far)-OMe, EDC, HOBt, CH_2Cl_2, 0 °C→RT, 89% over two steps; n) $[Pd(PPh_3)_4]$, DMB, THF, 65%; o) HCl/Et_2O, quantitative; p) AlocCl, NEt_3, CH_2Cl_2, 84%; q) TFA/CH_2Cl_2 (1:1), quantitative; r) H-Cys(Far)-OMe, EDC, HOBt, CH_2Cl_2, 0 °C→RT, 89%; s) $[Pd(PPh_3)_4]$, DMB, THF, 80%. Tol = tolyl, Ms = mesyl = methane sulfonyl, DMF = *N*,*N*-dimethylformamide, THF = tetrahydrofuran, DMA = *N*,*N*-dimethylacetamide, Aloc = allyloxycarbonyl, TFA = trifluoroacetic acid, Far = farnesyl, EDC = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide, HOBt = 1-hydroxy-1*H*-benzotriazole, DMB = dimethylbarbituric acid.

zepinedione core[9] was chosen as the underlying scaffold and equipped with a hydrolysis-stable sulfonamide to mimic the tetrahedral intermediate passed in the hydrolysis of the thioester (see **10**, Scheme 1).

For the synthesis of the desired compounds, 4-(*R*)-hydroxyproline and 5-bromoisatoic acid anhydride were condensed to give the benzodiazepinedione framework **2**, which was subjected to a ten-step synthesis sequence yielding central intermediates **5** and **6**. Methanesulfonamide **5** was converted into *N*-acylated and *S*-farnesylated cysteine methyl ester **7**. The *tert*-butyl ester was cleaved from hexadecylsulfonamide **6**, thereby giving rise to acid **8**. Alternatively, the amino group was masked and *S*-farnesylated cysteine methyl ester was attached to the C terminus to yield **9**, which was selectively *N*-deprotected to give benzodiazepinedione **10**, a mimic of the C terminus of fully processed H-Ras.

For determining the APT1-inhibiting potency of the synthetic benzodiazepinediones, a fluorescence-based biochemical assay was developed by employing the acrylodated intestinal fatty acid binding protein (ADIFAB) as the detecting system.[10]

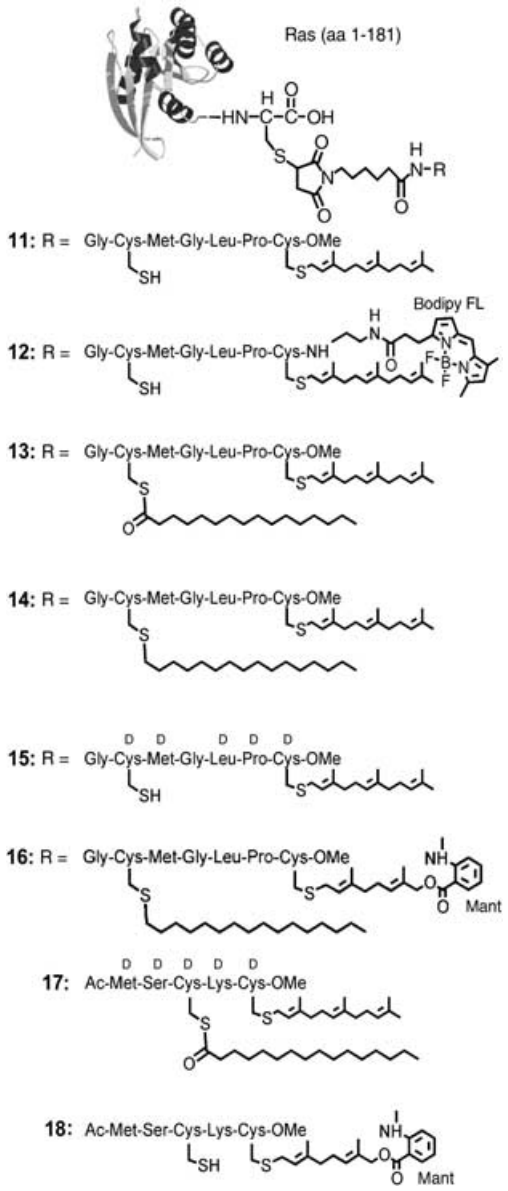
The results of the biochemical assay are shown in Table 1 (for additional data, see the Supporting Information). Compound **10** proved to be the most potent APT1 inhibitor with

Table 1: Inhibition of APT1 by benzodiazepinediones **5**–**10**.

Compound	IC_{50} [nM]
10	27 ± 5
9	149 ± 30
6	148 ± 6
8	97 ± 8
7	27 000 ± 17 000
5	250 000 ± 160 000

an IC_{50} value of 27 nM. The data indicate that a hexadecylsulfonamide moiety mimicking the palmitic acid thioester is required for high inhibitory activity (compare **7** and **10** or **5** and **6**). The primary amine moiety representing the ε-amino group of the lysine residue found at the C terminus of H-Ras should be liberated to obtain full inhibitory activity (compare **9** and **10**). An *S*-farnesylated cysteine methyl ester at the C terminus is beneficial for activity but not as essential as the palmitic acid mimic (compare **7**, **8**, and **10**). If both lipid residues are lacking, inhibitory activity is lost, that is, the benzodiazepinedione core is not sufficient for activity. The investigated compounds were not inhibitors of the enzyme protein farnesyltransferase (data not shown).

The biological activity of the APT1 inhibitors was investigated by employing the rat pheochromocytoma cell line PC-12. Under normal growth conditions, this cell line has a chromaffin-cell-like morphology. Upon microinjection of full-length recombinant oncogenic RasG12V proteins, these cells differentiate into nonreplicating sympathetic neuron-like cells.[11] The same morphological change is observed upon microinjection of semisynthetic oncogenic Ras protein **11** (Scheme 2), which carries an *S*-farnesylated cysteine methyl ester and a second unmasked, and therefore palmitoylatable,



Scheme 2. Structures of the semisynthetic Ras proteins **11**–**16** and the synthetic lipopeptides **17** and **18**; bodipy FL = 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl, mant = *N*-methylanthranilate.

cysteine residue at the C terminus.[12–14] In both cases lipidation of Ras is completed by *S*-palmitoylation in the cell, that is, protein **11** is converted into protein **13**, thereby resulting in plasma-membrane localization of and active signaling by the Ras proteins,[14] which is quantifiable by determining the frequency of neurite outgrowth in PC-12 cells. Microinjection of farnesylated but not *S*-palmitoylatable synthetic Ras (because of omission of a second cysteine in the synthesis) does not lead to neurite outgrowth. Consequently, a change in neurite outgrowth correlates directly with the degree of Ras palmitoylation.

Microinjection of synthetic, palmitoylatable N-Ras protein **11** was adjusted to give a neurite outgrowth rate of approximately 50% (see Figure 1). Depalmitoylation of Ras

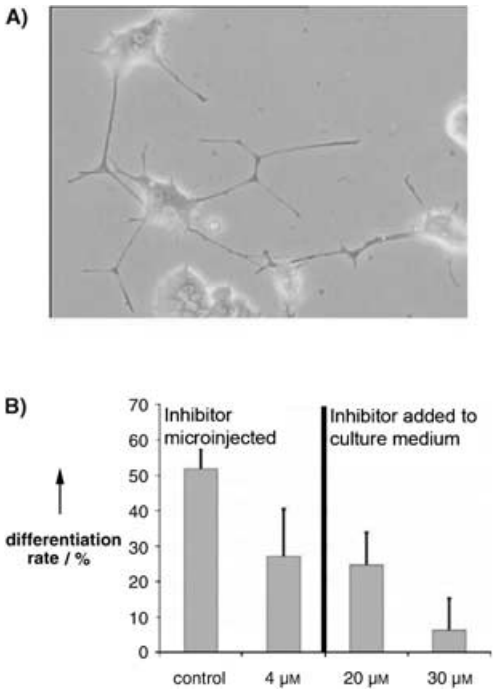


Figure 1. Reduction of PC-12 cell differentiation rate by APT1 inhibitor **6**. PC-12 differentiation assays were performed according to the procedure of Bader et al.[12] A 50 μM solution of the palmitoylatable lipopeptide coupling product of N-RasG12V(1–181) and **11** was microinjected into cells, thereby inducing a neurite outgrowth in approximately 50% of the injected cells (A). If APT1 inhibitor **6** was either coinjected (4 μM stock solution) with the Ras lipoprotein or added to the medium 1 h before microinjection (20 and 30 μM), the differentiation rate decreased significantly (B). For each experiment, an average of 100 cells were treated.

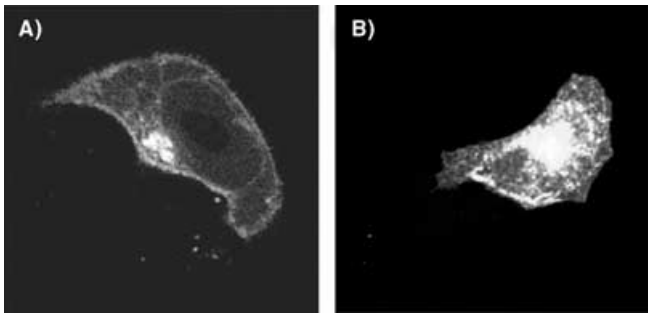


Figure 2. Inhibition of plasma-membrane localization of fluorescently labeled Ras protein **12** by APT1 inhibitor **6**. A 40 μM solution of the coupling product of N-RasG12V(1–181) and lipopeptide **12** was microinjected into MDCK cells. Localization of the fluorescent lipoprotein was monitored 7 h after microinjection by confocal microscopy. Although Ras protein alone shows a distinct staining of the plasma membrane (A), coinjection of 2 μM inhibitor **6** results in an accumulation of the lipoprotein in cytoplasmic structures, which is typical for nonpalmitoylatable Ras constructs (B).

by APT1 should result in reduced membrane binding of the oncogenic proteins[12,14] and in a reduction of neurite outgrowth. Therefore, upon inhibition of this process, an increased differentiation rate was to be expected.

Surprisingly, however, coinjection of a 4 μM solution or addition of APT1 inhibitor **6** to the culture medium (final concentration 20 μM) 1 h before microinjection of the Ras protein resulted in a *decrease* in the neurite outgrowth rate from above 50 to below 30%. An increase in the inhibitor concentration in the medium to 30 μM reduced the neurite outgrowth rate to a value below 10% (Figure 1).

To ascertain that the reduction in proliferation rate correlates with a lack of localization of Ras at the plasma membrane and consequently with inhibition of *S*-palmitoylation, synthetic fluorescent *S*-farnesylated and *S*-palmitoylatable N-Ras protein **12** was coinjected with compound **6** into MDCK cells. Confocal fluorescence microscopy revealed that the fluorescent protein had accumulated in cytoplasmic structures and the Golgi (Figure 2). In the absence of inhibitor, localization of the protein to the plasma membrane was clearly observed. When the corresponding fluorescent Ras protein in which the palmitoylatable cysteine residue was replaced by a nonpalmitoylatable serine residue was microinjected into Madin–Darby Canine Kidney (MDCK) cells, the protein was also localized in the cytoplasm and the Golgi (not shown).

When Ras protein **14**, which incorporates a hydrolysis-resistant hexadecyl thioether, was coinjected with inhibitor **6** at the same concentration as the palmitoylatable Ras protein had been, no reduction in neurite outgrowth was observed, as compared to the results after microinjection of lipidated protein **14** alone (not shown).

Microinjection of protein **15**, which carries only D-amino acids at the C terminus, into PC-12 cells surprisingly induced a differentiation rate that was comparable to the rate induced by the protein containing the natural L-amino acids.[14] This result indicates that the enzyme catalyzing the *S*-palmitoylation reaction must be fairly stereotolerant. To determine whether APT1 fulfills this criterion, the H-Ras-derived D-peptide **17** and the corresponding L-peptide were synthesized and subjected to depalmitoylation by APT1. The enzyme depalmitoylates both compounds with nearly identical rates (for the corresponding data, see the Supporting Information).

These results indicate that benzodiazepinedione **6** specifically inhibits Ras localization to the plasma membrane in the cells and, consequently, inhibits Ras palmitoylation rather than depalmitoylation. The fact that compound **6** displays an IC_{50} value for APT1 in the nanomolar range and that in the cells that were used for the microinjection experiments only *S*-palmitoylation remained to complete all the steps of posttranslational Ras modification required for plasma-membrane localization and induction of neurite outgrowth suggested that the observed effect might be linked to the inhibition of processes mediated by APT1. This includes the possibility that APT1 might be involved directly in Ras palmitoylation and in further downstream events that lead to Ras acylation by a different mechanism. We also cannot exclude the possibility that the effects described above are due to inhibition of other enzymes by benzodiazepinedione **6**.

To investigate whether APT1, in principle, can function as an *S*-palmitoylating enzyme in vitro, fluorescent palmitoylable peptide **18**, which represents the characteristic C termi-

nus of the H-Ras protein and incorporates a geranyl-*N*-mant function as a fluorescent substitute for the farnesyl group,[13] was treated with APT1 in the presence of palmitic acid. The palmitoylation reaction was monitored spectroscopically since conversion of the substrate **18** into the palmitic acid thioester results in a diagnostic shift of the fluorescence emission maximum from 440 to 410 nm, as determined by employing synthesized reference compounds (Figure 3).

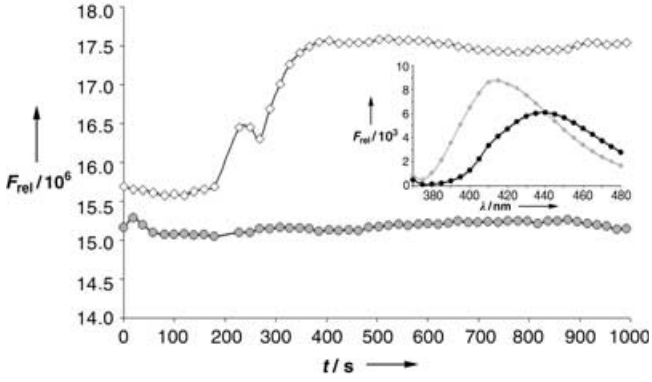


Figure 3. Palmitoylating activity of APT1 indicated by the change in relative fluorescence units (F_{rel}; measured at 410 nm) over time. Gray circles: control, that is, H-Ras peptide **18** and palmitic acid without APT1; white diamonds: H-Ras peptide **18**, palmitic acid, and added APT1. Conditions were as described in the Supporting Information). For better solubility of the palmitic acid, 10% dimethylsulfoxide (DMSO) was added to the reaction mixture. The concentrations for substrate **18**, APT1, and palmitic acid were 1, 1, and 100 μM, respectively. After the reaction mixture was incubated for 13 min, the reaction was started by addition of APT1. The result of a representative experiment is shown. Each data point represents the average of two independent measurements. The experiment was repeated six times with similar results (not shown). Small inset: emission spectra (370–480 nm) of palmitoylated (gray diamonds) and depalmitoylated (black circles) substrate peptide upon excitation at 360 nm.

Addition of APT1 to the reaction mixture led to a significant increase in fluorescence at 410 nm, thereby indicating *S*-palmitoylation of the substrate. The formation of the *S*-palmitoylated peptide was additionally confirmed by HPLC analysis of the reaction mixture and MALDI mass spectrometry (data not shown).[15]

A similar shift in fluorescence emission maximum was also observed when APT1 and palmitoyl coenzyme A were incubated with the synthetic, palmitoylatable N-Ras lipoprotein **16**, which again incorporates a fluorescent geranyl-mant instead of the farnesyl group.[13] Ras lipoprotein and palmitoyl donor or enzyme alone did not result in a shift of the emission maximum (see the Supporting Information). We utilized the hypsochromic effect to test whether inhibitors of APT1 were capable of preventing the enzyme-catalyzed palmitoylation of the Ras lipoprotein. Indeed, addition of compound **8** resulted in a clear blocking of the APT1-mediated shift in the fluorescence emission of the N-Ras construct (Figure 4).

To further determine whether APT1 does palmitoylate N-Ras in vitro, the protein was incubated with APT1 and [9, 10-^{3}H]-palmitic acid and the incorporation of radioactivity was

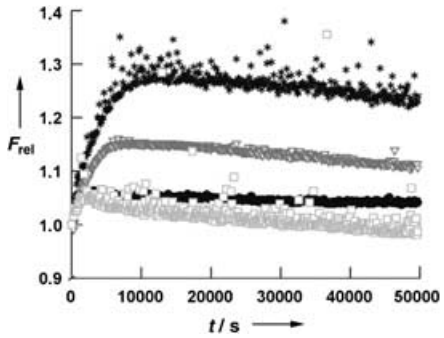


Figure 4. The APT1-mediated *S*-palmitoylation of N-Ras lipoprotein **16** employing palmitoyl coenzyme A (CoA) is inhibited by compound **8.** The change in relative fluorescence emission at 400 nm (F_{rel}) was measured under continuous stirring as a function of time. The concentrations of substrate **16**, APT1, and palmitoyl CoA were 500 nM, 50 nM, and 50 μM, respectively. After the reaction mixture was incubated for 15 min in the presence of different inhibitor concentrations, the reaction was started by addition of the enzyme. The result of one representative set of experiments (out of three) is shown. The palmitoylating reaction was studied in the absence of inhibitor (stars) or upon addition of compound **8** to the reaction mixture (triangles: 20 μM; circles: 40 μM; squares: 60 μM). The excitation wavelength was 360 nm. Measurements were performed at 20°C in 20 mM Hepes buffer (pH 7.4), 150 mM NaCl, 5 mM KCl, and 1 mM Na_2HPO_4. Palmitoyl CoA was stored at −20°C in aqueous solution containing 5 mM Na_2HPO_4. In each reaction mixture, the content of DMSO used as a solvent for compound **8** was adjusted to 0.3%.

determined by scintillation counting. In three separate experiments, the activity determined for the bands corresponding to the Ras protein in sodium dodecylsulfate PAGE was approximately 100% above the background value, thereby proving that N-Ras was palmitoylated (see the Supporting Information).

These results demonstrate that APT1 cannot only mediate Ras depalmitoylation but also Ras palmitoylation in vitro. Since the compounds shown in Table 1 may therefore be regarded as Ras-palmitoylation inhibitors we would like to refer to them as Raspalins 1–6 (according to the entries in the table, that is, compound **6** is termed Raspalin 3).

Enzymatic acylation by hydrolase-catalyzed reversal of the hydrolysis reaction, including that for plasma-membrane constituents,[16] is well-known[17] and incorporation of [^{3}H]-palmitic acid into N-Ras-derived farnesylated peptides in CV-1 cells has been noticed before.[18] In particular, Schuchman, Sandhoff, and co-workers convincingly demonstrated that acid ceramidase can catalyze ceramide synthesis in vitro and in vivo from free fatty acids and sphingosine by reverse hydrolysis, thereby providing a "salvage" pathway for ceramide biosynthesis.[16]

In addition, very recently Rando and co-workers reported that the enzyme lecithin retinal acyl transferase (LRAT) catalyzes the reversible interconversion of all-*trans*-retinal, *trans*-retinyl palmitate, and the triply *S*-palmitoylated membrane-associated protein mRPE65.[19]

The notion that APT1 may not only function as a thioesterase but also as an acyltransferase is furthermore supported by the fact that the nucleophilic serine residue 114

that forms part of the catalytic Ser-His-Asp triad of the protein is located within a Gly-Xaa-Ser-Xaa-Gly motif (Gly112-Phe113-Ser114-Glu115-Gly116). This motif has previously been identified as typical for enzymes with acyltransferase and thioesterase activity[20] and proven to be of diagnostic and predictive value.[21] Finally, we note that the broad substrate tolerance displayed by APT1 would also explain the finding that a consensus palmitoylation peptide sequence motif does not seem to exist.[4]

Our results demonstrate that the thioesterase APT1 can depalmitoylate and palmitoylate the Ras protein in vitro. From these observations a direct involvement of APT1 in Ras palmitoylation in vivo cannot be conclusively delineated. However, this possibility exists. Future in-depth evaluation of the biological role of APT1 by biology and chemical biology techniques is required to prove such a hypothesis.

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[15] The enzymatic synthesis of a thioester from a carboxylic acid and a thiol a priori is an energetically uphill process; in this respect, it is comparable to the enzymatic synthesis of a peptide from the thermodynamically more favorable amine. The principle by which a thioesterase can catalyze the formation of a thioester from a thiol and an acid can therefore be regarded as analogous to peptide synthesis by proteases if both enzyme types employ the same mechanism. APT1 is a cysteine hydrolase displaying the same catalytically active triad as cysteine and serine proteases; the enzymes chymotrypsin (serine protease) and papain (cysteine protease) can serve as analogous cases. For these enzymes, it has been demonstrated that catalysis of the synthesis reaction is achieved by lowering the transition state of the transformation through entropic and enthalpic contributions. Thus, the enzymes concentrate and correctly orientate the substrate and restrict the mobility of the groups concerned with the transition state, thereby causing changes in activation entropy. In addition, charge distinction in the active site and the geometry of substrate binding are changed, which affects the activation enthalpy. Furthermore, in the presence of solvents other than water and in the presence of a second phase, criteria that both are fulfilled in a membraneous environment, the hydration of ionic groups, most notably carboxy functions, is diminished, thereby making them more prone to nucleophilic attack. Also, the formed product, which is more hydrophilic than the starting materials, is sequestered away under these conditions, particularly in a two-phase system, for example, at a water/membrane interface. The free-energy change of this transfer also provides a driving force for synthesis. In addition, the decision between deacylation of an intermediary acyl–enzyme complex by water (that is, hydrolysis) and acyl transfer to a different nucleophile like an amine or a thiol (that is, synthesis) is strongly influenced by the nucleophilic strength of the competitors. In this respect, amines and, even more so, thiols have an unequivocal advantage over water. For instance, leucine amide (0.25 M) deacylates an acyl–chymotrypsin complex 20 times faster than water (55 M); see: K. Morihara, T. Oka, *Biochem. J.* **1977**, *163*, 531.
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