

Discovery and characterization of inhibitors of human palmitoyl acyltransferases

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Abstract

The covalent attachment of palmitate to specific proteins by the action of palmitoyl acyltransferases (PAT) plays critical roles in the biological activities of several oncoproteins. Two PAT activities are expressed by human cells: type 1 PATs that modify the farnesyl-dependent palmitoylation motif found in H- and N-Ras, and type 2 PATs that modify the myristoyl-dependent palmitoylation motif found in the Src family of tyrosine kinases. We have previously shown that the type 1 PAT HIP14 causes cellular transformation. In the current study, we show that mRNA encoding HIP14 is up-regulated in a number of types of human tumors. To assess the potential of HIP14 and other PATs as targets for new anticancer drugs, we developed three cell-based assays suitable for high-throughput screening to identify inhibitors of these enzymes. Using these screens, five chemotypes, with activity toward either type 1 or type 2 PAT activity, were identified. The activity of the hits were confirmed using assays that quantify the *in vitro* inhibition of PAT activity, as well as a cell-based assay that determines the abilities of the compounds to prevent the localization of palmitoylated green fluorescent proteins to the plasma membrane. Representative compounds from each chemotype showed broad antiproliferative activity toward a panel of human tumor cell lines and inhibited the growth of tumors *in vivo*. Together, these data show that PATs, and HIP14 in

particular, are interesting new targets for anticancer compounds, and that small molecules with such activity can be identified by high-throughput screening. [Mol Cancer Ther 2006;5(7):1647–59]

Introduction

Numerous proteins involved in a variety of cellular processes are known to have lipids attached to them either cotranslationally or posttranslationally, which regulates their subcellular distribution and activities (1, 2). Myristoylation and isoprenylation involve irreversible attachment of lipids to proteins, whereas palmitoylation is a reversible modification with a relatively rapid turnover rate (3, 4). The reverse reaction is carried out by thioesterases that cleave the palmitate from proteins (5, 6). The main function of protein palmitoylation is to facilitate membrane binding of the modified proteins. Although other fatty acid modifications also increase protein hydrophobicity, those modifications result in only transient membrane association, with half-lives of <1 minute (7). Furthermore, proteins that undergo palmitoylation in conjunction with either N-myristoylation or C-prenylation show an even stronger membrane association with half-lives of >70 hours (8, 9). Numerous studies have shown that palmitoylation targets proteins to lipid rafts or caveolae in the plasma membrane (10, 11). These domains are enriched with signaling proteins (12–15) and are thought to play a role in signal transduction by effectively increasing the local concentrations of these signaling components.

Proteins known to be reversibly palmitoylated include certain Ras isoforms; many members of the Src family of protein tyrosine kinases; subunits of G proteins and G protein-coupled receptors; rhodopsin; and several neuron-specific proteins, such as GAP-43, SNAP-25, and PSD-95 (16–18). Members of this “palmitoylome” can be categorized into four groups based on their sites of palmitoylation (19, 20). Several proteins contain palmitoylation sites on cysteine residues near transmembrane or integral membrane domains, or on cysteine residues in COOH-terminal or NH₂-terminal regions. However, these proteins do not seem to contain specific palmitoylation sequences for recognition by palmitoyl acyltransferase (PAT) enzymes, and are probably palmitoylated by nonenzymatic reaction with palmitoyl-CoA. In contrast, two other classes of palmitoylated proteins are of special interest because they contain discrete recognition motifs for PAT enzymes. Class 1 proteins are first farnesylated and then palmitoylated. Examples of these proteins include the H, N, and K2A isoforms of Ras and paralemmin. For these proteins, farnesylation occurs on the cysteine residue of a COOH-terminal CAAX motif, and this is required for subsequent palmitoylation of one or more cysteine residues located near the farnesylcysteine (21). Therefore, it is likely that this

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Table 1. Primer pairs for yeast gene disruption

Name	Primer sequence	RE	PCR (bp)
<i>PDR1_5_5</i>	GAGACAATGGTTGACCTTTTGTCC	<i>Xma</i> I	1,066
<i>PDR1_5_3</i>	GACCTTGGCTTACGAATCTTGC	<i>Sac</i> II	
<i>PDR1_3_5</i>	caggccgcccGACCTCTACAGTATCCTGTGGAGCG	<i>Sac</i> II	976
<i>PDR1_3_3</i>	GCCCTTATACTGTCCAGTTGAGTAAGG	<i>Bam</i> HI	
<i>PDR3_5_5</i>	cgctagcCGTCTGATGTATCTCGATTTCG	<i>Nhe</i> I	1,088
<i>PDR3_5_3</i>	gcgaattcGCAGTTGGTACATGGATATTTACC	<i>Eco</i> RI	
<i>PDR3_3_5</i>	gcgaattcCAGTTGTATGACAGTACTGCTCC	<i>Eco</i> RI	1,089
<i>PDR3_3_3</i>	cgctcgagCTTGCACCTCAGCTTCCTC	<i>Xho</i> I	
<i>YOR1_5_5</i>	ccatcgatCTTTGACTGTTACCCGGTTG	<i>Cl</i> aI	1,074
<i>YOR1_5_3</i>	gcgaattcGAAGAAGCAGATGCCTTGG	<i>Eco</i> RI	
<i>YOR1_3_5</i>	gcgaattcATGTGTTCTAGATCTGGTATTGTGG	<i>Eco</i> RI	1,049
<i>YOR1_3_3</i>	cgctcgagCATACTCTGGTAAGCAAGTCGG	<i>Xho</i> I	
<i>RAS2_5_5</i>	cgctagcCAGCAACAACAGCAGGATATC	<i>Nhe</i> I	1,075
<i>RAS2_5_3</i>	gcgaattcGCAGATTACCAACACCACC	<i>Eco</i> RI	
<i>RAS2_3_5</i>	gcgaattcCTAAACAGGCTAGGAAGCAGC	<i>Eco</i> RI	1,026
<i>RAS2_3_3</i>	cgctcgagCACCTCTGCTACCAGTAAAGCG	<i>Xho</i> I	

COOH-terminal prenylation motif serves as a palmitoylation signal that is recognized by one or more PAT enzymes. The class 2 proteins are first N-myristoylated and then palmitoylated. Examples of these proteins include certain α subunits and several Src-related tyrosine kinases. These proteins contain a Met¹-Gly²-Cys³ sequence that is the site of N-myristoylation following cleavage of the initiator Met¹ by methionine aminopeptidase 2, and this is required for the palmitoylation of one or more cysteines near the N-myristoylglycine (9, 22, 23). This N-myristoylglycine motif also likely serves as a palmitoylation signal that is recognized by one or more PAT enzymes.

The class 1 and 2 proteins represent the two major categories of proteins with distinct palmitoylation motifs that are enzymatically modified, suggesting that multiple PAT enzymes exist to recognize these specific motifs, herein termed type 1 and type 2 PATs, respectively. We have described a sensitive *in vitro* palmitoylation assay that allows the analysis of the enzymatic palmitoylation of fluorescent peptides that mimic the two palmitoylation motifs (24, 25). Using this assay, it has been shown that membrane fractions from different cell lines have differential activities toward the two peptide substrates, and that the two types of PAT activities are differentially affected by various chemical treatments or changes in the assay parameters. Additionally, membranes isolated from wild-type NIH/3T3 cells showed significant PAT activity toward the myristoylated peptide, but have very little PAT activity toward the farnesylated peptide; on the other hand, Ras-transformed NIH/3T3 cells showed a significant increase in activity toward the Ras-mimetic substrate (26). These results, and those from yeast systems (27, 28), show that there are multiple PATs that recognize unique peptide substrates.

To date, no small-molecule inhibitors of PAT enzymes have been developed. This is largely due to the fact that the molecular identities of human PATs have not been

resolved, and candidates for these enzymes have only recently been found. In the present report, we show that HIP14, a type 1 PAT recently characterized in our laboratory (26) and another (29), is up-regulated in a number of cancer types compared with matching normal tissue. To investigate the role of HIP14 and other PAT enzymes in cancer development and progression, we have initiated a program to identify inhibitors of the PAT enzymes that have specificity for either the type 1 or type 2 palmitoylation motif. We have developed and implemented three cell-based screens designed to identify these inhibitors, and report here five chemotypes that possess anti-PAT activity. Four of the five chemotypes are selective for type 1 PATs, whereas the fifth is selective for type 2 PATs. In addition, we provide evidence that these compounds function in intact cells and have antitumor activity *in vivo*.

Materials and Methods

Constructs

Yeast Strains. Gene disruptions in the W303 yeast strain (*MAT α* [*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*] [*phi+*]) were carried out by the two-step method (30, 31). DNA sequences targeting disruptions to particular genes were cloned into a derivative of Yip5 (32), called DipDL. DipDL is modified to be a high-copy plasmid in *Escherichia coli* and carries a unique multiple cloning site.⁵ Sequences targeting ~1 kb of *PDR1*, *PDR3*, *YOR1*, and *RAS2* were PCR amplified using the primers listed in Table 1 and cloned into the DipDL vector using the restriction enzymes indicated in the table. Disruption plasmids were linearized and transformed into yeast. Ura⁺ transformants were

⁵J.D. Diller, unpublished data.

chosen for growth without selection, and then spread on plates containing 0.02% 5-fluoroorotic acid (31, 33). The YCD4 strain genotype: *MATa/MAT α* {*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*} [*phi+*] Δ *PDRI*, Δ *PDRI3*, Δ *YOR1*; YCD5 strain genotype: {*leu2-3,112 trp1-1 can1100 ura3-1 ade2-1 his3-11,15*} [*phi+*] Δ *PDRI*, Δ *PDRI3*, Δ *YOR1*, Δ *RAS2*.

Green Fluorescent Protein. The C-farn-palm-GFP (pEGFP-F) construct was generated by subcloning the RAS-GFP DNA (previously described in ref. 34) into the pTRE2hyg vector (BD Biosciences, San Jose, CA). The vector contains the COOH-termini of wild-type H-Ras fused to the green fluorescent protein (GFP) under the control of the tetracycline operator. The N-myr-palm-GFP construct was generated by subcloning the Fyn-GFP DNA (previously described in ref. 34) into the pTRE2hyg vector (BD Biosciences). This construct contains the NH₂-terminal myristoylation signal of Fyn fused in frame to the NH₂ terminus of GFP under the control of the tetracycline operator. All constructs were verified by sequencing before use.

Northern Blot Analysis

The Cancer Profiling Array II was obtained from BD Biosciences. The membrane was hybridized with a 498 bp HIP14 probe, generated by PCR, and a ubiquitin control cDNA provided by the manufacturer. All probes were random prime labeled with [α -³²P]dATP to a specific activity of 10⁸ cpm/ μ g. The membranes were exposed to a FujiFilm phosphorimager cassette and read in the Typhoon 9410 phosphorimager (GE Healthcare, Piscataway, NJ). Spots were quantified using ImageQuant software and the relative expression levels were calculated using the ubiquitin signal as the control.

Drug Accumulation by Yeast

Yeast were grown to an *A*₆₀₀ of 1.0, and 0.8 mL was added to an Eppendorf tube. 0.1 μ Ci of [³H]drug (i.e., [³H]vinblastine, [³H]Taxol, [³H]ritinovir, [³H]actinomycin D, [³H]clozapine, or [³H]daunomycin) was added to each tube and incubated for 60 minutes at 30°C. The samples were then centrifuged for 5 minutes at 1,900 \times *g* at 4°C. Radioactive medium was aspirated and the yeast were washed with 1 mL of ice-cold PBS. The yeast were again centrifuged, the PBS was aspirated, and 0.6 mL of 1% SDS was added. Cell lysates were collected and the amount of [³H]drug accumulated by the cells was quantified by scintillation counting.

GFP Displacement Assay

293 Tet-on cells (Clontech, Mountain View, CA) were stably transfected with C-farn-palm-GFP and N-myr-palm-GFP expression constructs under the control of the tet-operator. Stable clones were plated in 24-well plates, grown for 24 hours, treated with the 25 μ g/mL of a test compound for 0.5 hours, and induced with 1 μ g/mL doxycycline. The localization of the GFP was visualized by fluorescence microscopy 24 to 48 hours postinduction.

Cell Culture

MCF-7, HepG2, T-24, SK-OV-3, Caco-2, Du145, Panc-1, MDA-MB-231, and A-489 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were

maintained in either RPMI 1640 or DMEM containing 10% FCS, 50 μ g/mL gentamicin, and 1 mmol/L sodium pyruvate at 37°C in an atmosphere of 5% CO₂ and 95% air. All tissue culture reagents were from Life Technologies (Carlsbad, CA).

In vitro Palmitoylation Assay

Membranes from MCF-7 cells were prepared, and the *in vitro* palmitoylation assay was conducted as previously described (24, 25). The fluorescent peptides (NBD)-CLC(OMe)-Farn and Myr-GC(NBD) were synthesized by solution-phase chemistry using mild conditions to maintain chemically labile functional groups, e.g., the farnesyl-cysteine thioether linkage (Fig. 1).⁶ The peptides were stored under argon at -80°C, and deprotected as previously described immediately before their use.

Western Blot Analysis

Cells were resuspended in lysis buffer [10 mmol/L HEPES (pH 7.4), 1% SDS]. The protein concentration in each sample was determined using the fluorescamine assay (35), and samples were normalized for equal amounts of protein (50-100 μ g/lane) were separated on 10% SDS-PAGE gels and electrotransferred to polyvinylidene difluoride membranes. The membranes were incubated in PBST or TBST [PBS: 100 mmol/L sodium phosphate, 100 mmol/L sodium chloride (pH 7.6); or TBS: 20 mmol/L Tris base, 137 mmol/L sodium chloride (pH 7.6) containing 0.1% Tween 20] plus 5% powdered milk (w/v) at room temperature for 2 hours. For the phosphospecific blots, the membranes were washed thrice in TBST at room temperature for 5 minutes. Membranes were then incubated with anti-phospho-Mek1/2 (Cell Signaling, Danvers, MA) in TBST plus 5% bovine serum albumin on a shaking platform for 18 hours at 4°C. The membranes were washed thrice in TBST for 10 minutes each at room temperature, and antirabbit antibodies conjugated to horseradish peroxidase (Sigma, St. Louis, MO) were then incubated with the membranes in TBST containing 3% powdered milk (w/v) on a shaking platform for 1 hour at room temperature. The blots were washed four times in TBST for 5 minutes each at room temperature, and then developed with SuperSignal reagents (Pierce, Rockford, IL). Filters were stripped of the original antibody by incubation in Restore Western blot stripping buffer (Pierce) then reprobed with antibodies against total Mek 1/2 (Cell Signaling). Blots were imaged with a FUJIFILM Intelligent Dark Box II, and the appropriate bands were quantified using Image Gauge 4.0 software.

In vivo Tumor Growth

The *in vivo* activities of the PAT inhibitors were tested in a syngeneic mouse tumor model that uses the JC murine mammary adenocarcinoma cell line growing in BALB/c mice (36). Female BALB/c mice, 6 to 8 weeks old, were injected s.c. with 1 \times 10⁶ JC cells suspended in PBS. After palpable tumor growth, tumor volumes were determined

⁶Z. Xia and C.D. Smith. Synthesis of fluorescent peptide substrates for cellular palmitoyl acyltransferase, in preparation.

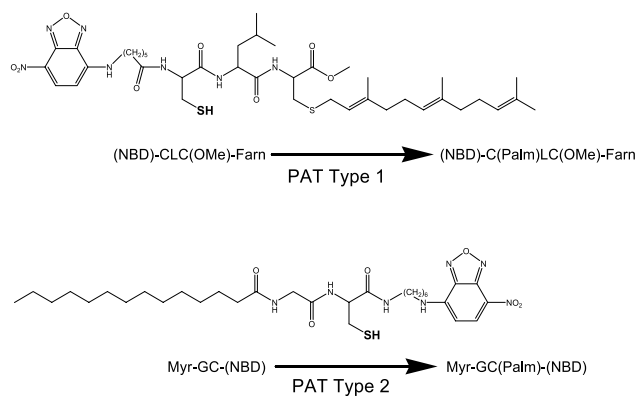


Figure 1. Palmitoylation motifs recognized by PATs. (NBD)-CLC(OMe)-Farn contains the COOH-terminal farnesylated cysteine and an upstream palmitoylatable cysteine found in proteins such as N-Ras and H-Ras. Myr-GC(NBD) mimics proteins with an NH₂-terminal myristoylated glycine followed by a palmitoylatable cysteine residue, mimicking Src-related tyrosine kinases.

(day 1) using calipers to measure the length (L) and width (W) of the tumor and the equation $(L \times W)/2$. Each treatment group contained five mice. Treatment consisted of i.p. administration of either 30 μ L DMSO (control) or a test PAT inhibitor in DMSO at a final dose of 25 mg/kg. Drugs were administered daily for 5 days, and then the mice were allowed to recover for 2 days and given an additional of five daily doses. Whole body weight and tumor volume measurements were done on days 1, 5, 8, 10, 12, and 15. Increases in tumor volume were compared using the Bonferroni multiple comparisons test.

Results

Expression of HIP14 in Human Tumors

We previously showed that HIP14 has PAT activity, with selectivity for the farnesyl-dependent palmitoylation motif found in H- and N-Ras proteins, and can directly transform cells (26). Northern and Western analyses of HIP14 have shown that it is expressed in most human and mouse tissues (37). To determine whether HIP14 is overexpressed in human tumors compared with normal tissue, the Cancer Profiling Array II was hybridized with a radiolabeled HIP14 probe. This array contains cDNA pairs from matched normal and tumor samples isolated from 19 different tissues from a total of 154 patients. The samples were normalized by hybridizing the array with a ubiquitin control cDNA provided by the manufacturer. An increase in HIP14 message of 50% above normal tissue was set as the minimal increase for a sample to be considered up-regulated, and a decrease of >20% was set as the cutoff for message down-regulation. The expression pattern of HIP14 mRNA in these samples is indicated in Fig. 2. The data indicate that HIP14 message is up-regulated in 70% of patients with colon, stomach, and breast tumors; 60% of lung tumors; and 50% of prostate tumors. Interestingly, the level of HIP14 message in these tissues generally followed

with the increase in gradation of the tumor stage (where stage information was available). For example, stage I, II, and III breast tumors had average increases of 52%, 64%, and 230%, respectively. In the colon, HIP14 message was not increased in stage I tumors, whereas stage II and III tumors had an average increase of 82% and 87%, respectively, and the single stage IV tumor was increased by 120%. A similar trend was seen with stomach tumors. These results suggest that HIP14 levels may be a new genetic marker for the progression of these diseases; however, this clearly needs to be confirmed with larger numbers of patient samples. Nonetheless, the increase in HIP14 message expression with disease progression, in conjunction with our observations that HIP14 can function as an oncogene, are indicators that PAT inhibitors should be effective agents for the treatment of several types of cancer. Interestingly, HIP14 message levels were significantly reduced in all of three liver cancer specimens and the majority of cancers of the pancreas, thyroid gland, bladder, and vulva (Fig. 2B).

PAT Inhibitor Screens

Three different screening assays were used to discover compounds that inhibit PAT activity. Two of these assays were designed to identify inhibitors of PATs with the farnesyl-peptide specificity (type 1), and one assay was designed to identify inhibitors of PATs with the myristoyl-peptide specificity (type 2). Compounds screened were from the DIVERSet collection available from the Chem-Bridge Corporation (San Diego, CA). DIVERSet is a unique collection of drug-like, hand-synthesized small molecules, rationally selected to form a "universal" library that covers the maximum pharmacophore diversity with the minimum number of compounds. These compounds are described in detail online.⁷

Screen 1. HIP14 Inhibitor Screen. Overexpression of HIP14 in NIH/3T3 fibroblasts results in marked phenotypic changes, including the loss of serum dependence for proliferation, the formation of foci when grown on plastic, the formation of colonies when grown in soft agar, and the ability to form tumors in mice (26). Use of RNA interference to deplete HIP14 from HIP14-transformed cells or H-Ras-transformed cells resulted in a dramatic slowing of growth and DNA replication compared with wild-type cells.⁸ This is consistent with other studies that show that cells typically become dependent on the continued overexpression of a transforming oncoprotein. In light of the small interfering RNA result and the fact that the growth of both cell types was similar in the presence of DMSO, we have used the HIP14-overexpressing 3T3 cells to screen for inhibitors of HIP14 activity. The screen compares the growth of the HIP14-overexpressing 3T3 cells to the growth of wild-type NIH/3T3 cells in the presence of compounds from our libraries. As with the

⁷ <http://chembridge.com/chembridge/compound.html>

⁸ C.E. Ducker, et al. HIP14 directly affects Ras-mediated transformation, submitted for publication.

PAT inhibitor allows the strain to return to a normal growth rate, and when YCD5 cells were removed from 2-bromopalmitate, they recovered and proliferated at the same rate as YCD4 (data not shown).

For the screening assays, YCD4 and YCD5 cells were plated in 96-well microtiter plates at a starting A_{600} of 0.01, and grown in the presence of a screening compound for 14 hours before the final A_{600} reading was taken. A YCD4/YCD5 ratio of ≥ 10 was considered a positive hit, as long as YCD4 grew to an A_{600} of 1.0 ± 0.2 , i.e., the compounds are not cytotoxic by a non-PAT-mediated mechanism. Approximately 8,000 were tested in this assay, with a hit rate of 0.4%. Hits were confirmed by retesting in the screening assay and using the standard *in vitro* palmitoylation assay

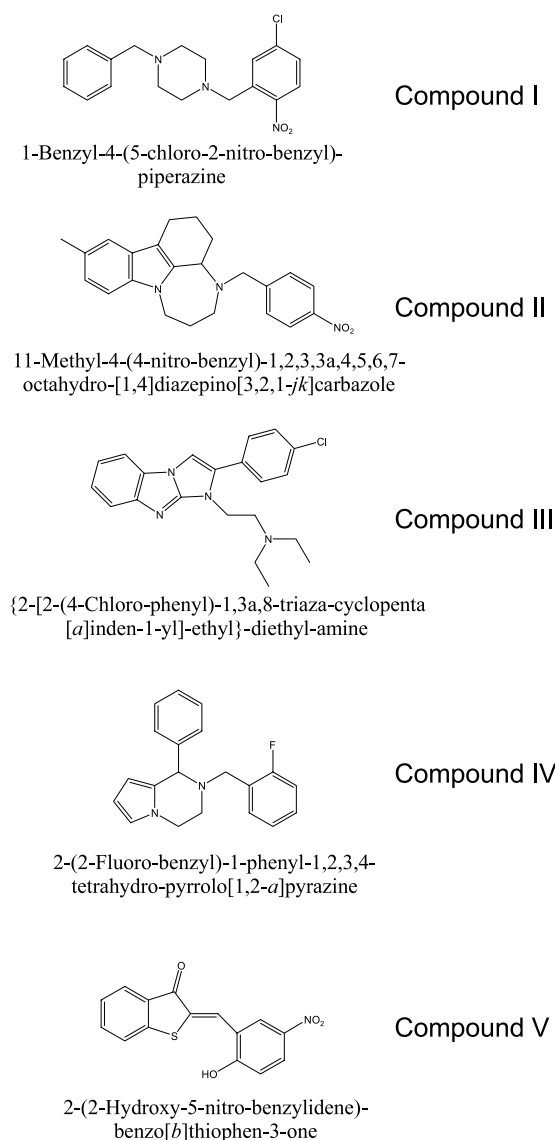


Figure 3. Representative PAT inhibitors from each of the five chemotypes identified by screening. Structures and chemical names for compounds I to V.

(24, 25). One chemotype, represented by compound IV of Fig. 3, comprised 6 of the 32 confirmed hits in this screen. In addition, our laboratory has synthesized ~30 analogues within this series that also inhibit growth in this screening assay and reduced PAT activity in the *in vitro* palmitoylation assay.⁹

Screen 3. Myristoyl-Peptide Screen. Similar to previously described substrates for type 2 PATs (38), the fluorescent peptide Myr-GC(NBD) is taken up by cells and quickly palmitoylated, causing the cells to fluoresce (Fig. 1).⁶ Blocking the palmitoylation of this peptide reduces the fluorescence of the cells by reducing the association of the NBD moiety with the plasma membrane. This phenomenon is readily seen by fluorescence microscopy¹⁰ and can be quantified using a fluorescence-capable plate reader (excitation 485 nm; emission 535 nm). In the screening assays, Jurkat cells were plated at a density of 10^5 cells/mL in 96-well plates, treated with a screening compound, and incubated with the labeled peptide for 2 hours. The fluorescence signal was then quantified using a Perkin-Elmer HTS Plus plate reader (Perkin-Elmer, Shelton, CT). Compounds were considered hits if they reduced the fluorescence by $\geq 50\%$. Approximately 16,600 compounds were tested in this assay, with a hit rate of 0.2%. Hits were confirmed by retesting in the screening assay and using the standard *in vitro* palmitoylation assay (24, 25). One chemotype, represented by compound V of Fig. 3, was identified by this screen, and comprised 4 of the 33 confirmed hits.

In summary, we have identified five unique chemotypes that inhibit type 1 and/or type 2 PAT activity using these new screening assays. The compounds depicted in Fig. 3 represent these chemotypes, and *in vitro* and *in vivo* characterization of these compounds (described below) shows that these chemotypes have good potency as PAT inhibitors, provide selective inhibitors for type 1 and type 2 PATs, and can function in intact cells.

Specificity of Compounds for Type 1 and Type 2 PATs

Each compound that met the criteria for a hit in one of the screens was confirmed by demonstrating its ability to inhibit PAT activity *in vitro* (24, 25). These assays assess the effects of the compounds on the *in vitro* palmitoylation of fluorescent peptides that mimic the COOH-terminal farnesyl-palmitoylation motif (NBD)-CLC(OMe)-Farn, or the NH₂-terminal myristoyl-palmitoylation motif, Myr-GC(NBD) (Fig. 1). All confirmed hits from the screens were tested for their ability to inhibit palmitoylation of both peptide substrates at a single high dose (25 $\mu\text{g}/\text{mL}$, ~30–40 $\mu\text{mol}/\text{L}$), and one compound from each chemotype was further characterized (summarized in Table 2). Several compounds showed good selectivity for either type 1 or

⁹ Y. Zhuang, et al. Synthesis and evaluation of substituted cyclohexyl-octahydro-pyrrolo[1,2-a]pyrazines that inhibit palmitoyl acyltransferases, in preparation.

¹⁰ J.M. Draper, et al. Cellular analyses of protein palmitoyl acyltransferases, submitted for publication.

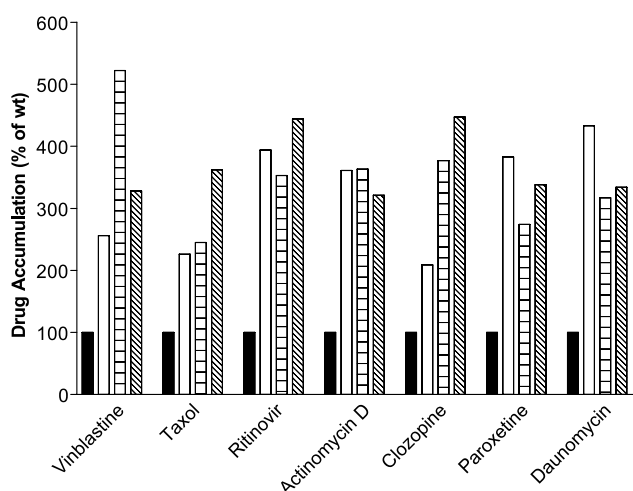


Figure 4. Intracellular drug accumulation by YCD4 clones. YCD clones 4-1 (white columns), 4-2 (horizontal-hatched columns), 4-4 (cross-hatched columns), and wild-type W303 (black columns) cells were grown to an A_{600} of 1.0. Each type of cell was then incubated with 0.1 μ Ci of either vinblastine, Taxol, Ritinovir, actinomycin D, clozopine, or daunomycin for 1 h at 30°C. Cells were collected and washed twice in ice-cold PBS. Cells were resuspended in 1% SDS and the amount of 3 H in the cells was quantified by scintillation counting. Data are normalized to drug levels in wild-type cells, which was set to 100%.

type 2 PAT in the *in vitro* palmitoylation assay using either the (NBD)-CLC(OMe)-Farn or Myr-GC(NBD) peptide (Table 2). The data indicate that compounds I, II, III, and IV are selective inhibitors of type 1 PAT activity, whereas compound V is a selective inhibitor of type 2 PAT activity. This isozyme selectivity is highly desirable because it is likely that compounds that selectively target type 1 PAT activity will have fewer undesired effects. This is because there are very few proteins, other than the Ras proteins that are being targeted, that contain the COOH-terminal farnesyl-palmitoylation motif. Conversely, compounds that inhibit the NH₂-terminal myristoyl-palmitoylation motif may affect signaling through a variety of Src-related kinases, as well as G proteins.

Potency of PAT Inhibitors *In vitro*

Compounds I, II, III, IV, and V (at 25 μ g/mL) inhibited PAT activity by 75%, 78%, 78%, 72%, and 74%, respectively, and serve as prototypes for nonlipid PAT inhibitors. Although the screens provided rapid identification of compounds PAT inhibitors, additional studies were necessary to determine their potencies. Therefore, the effects of compounds I to V were determined at multiple concentrations in the *in vitro* palmitoylation assay (Fig. 5). As summarized in Table 2, the compounds showed IC₅₀ values in the low micromolar range, making them more potent inhibitors of PAT than any previously reported compound.

Activity of PAT Inhibitors in Intact Cells

Although the PAT inhibitors were active in the *in vitro* assays, it was important to determine their abilities to inhibit endogenous PATs in intact cells. Similar to our previously described reporter systems (26), we have now

developed cellular assays for type 1 and type 2 PAT inhibitors by generating HEK293 cells that are stably transfected with inducible systems for the expression of GFPs containing either the NH₂-terminal sequence of Fyn, which directs it to be N-myristoylated and then palmitoylated (this construct is called N-myristoyl-palm-GFP) or the entire sequence of H-Ras, which directs it to be C-farnesylated and then palmitoylated (this construct is called C-farn-palm-GFP). In these experiments, 293 Tet-on cells were stably transfected with the C-farn-palm-GFP or N-myristoyl-palm-GFP expression constructs under the control of the tet-operator. Stable clones were plated in 24-well plates, grown for 24 hours, treated with the 25 μ g/mL of a PAT inhibitor for 0.5 hours, and induced with 1 μ g/mL doxycycline. The localization of the GFP was visualized by fluorescence microscopy 24 to 48 hours postinduction. Compounds I, II, III, or IV were tested in cells expressing the C-farn-palm-GFP construct, whereas compound V was tested in cells expressing the N-myristoyl-palm-GFP construct.

Expression of either GFP construct in cells treated with DMSO resulted in the localization of GFP at the cell periphery with punctuate foci along the cell-to-cell boundaries and little cytoplasmic fluorescence (Fig. 6, top), indicating that the dual lipidation reactions target the protein to the plasma membrane. Treatment of the cells with compound I or II increased the cytoplasmic fluorescence, but did not totally eliminate the peripheral membrane fluorescence. However, treatment with compound III, IV, or V dramatically increased the cytoplasmic fluorescence and totally eliminated the peripheral membrane fluorescence (Fig. 6). Interestingly, there is an accumulation of fluorescence around the nucleus of the cells and in subcellular vesicles, suggesting that the unpalmitoylated GFP proteins are accumulating in the Golgi and trans-Golgi network. These results indicate that the PAT inhibitors effectively penetrate the cells and can inhibit endogenous PAT activity. Furthermore, compounds III, IV, and V can totally eliminate plasma membrane binding of the GFP constructs, indicating that they should be able to neutralize the functions of palmitoylated proteins such as H-Ras and Fyn, which require proper subcellular localization for their actions.

Survey of Cell Line Sensitivity

The chemotherapeutic potencies of the representative compounds from the five chemotypes toward a panel of human adherent tumor cell lines representing several major tumor types were determined (Table 3). Compound V was typically the most potent inhibitor for the growth of all of the cell lines, with IC₅₀ values in the low micromolar range, except for SKOV3 and HepG2 cells that were less sensitive. The potencies of compounds I to IV varied from 3 to >145 μ mol/L. It is interesting to note that compound II was particularly potent toward breast cancer cell lines. Estrogen receptor-positive MCF7 cells were more sensitive to all of the PAT inhibitors than the estrogen receptor-negative MDA-MB-231 cells, and this is consistent with the role of Ras proteins in estrogen receptor signaling.

Table 2. Summary of *in vitro* properties of PAT inhibitors

Compound	<i>In vitro</i> palmitoylation-Farn (%inhibition at 25 µg/mL)	<i>In vitro</i> palmitoylation-Myr (%inhibition at 25 µg/mL)	<i>In vitro</i> palmitoylation IC ₅₀ (µmol/L)	Inhibition of membrane localization of GFP (at 25 µg/mL)
I	76	15	11.8 (Farn)	++ (GFP-Ras)
II	78	28	4.1 (Farn)	+ (GFP-Ras)
III	78	9	5.2 (Farn)	+++ (GFP-Ras)
IV	72	8	1.2 (Farn)	+++ (GFP-Ras)
V	17	74	0.5 (Myr)	+++ (GFP-Myr)

NOTE: Compounds I to V were analyzed using the *in vitro* palmitoylation assay with either the (NBD)-CLC(OMe)-Farn peptide (*in vitro* palmitoylation-Farn) or the Myr-GC(NBD) peptide (*in vitro* palmitoylation-Myr). Values for the percentage inhibition of PAT activity at 25 µg/mL and the IC₅₀ (µmol/L) are given. The effects of the compounds on the localization of cells expressing either the GFP-Ras construct or the GFP-Myr construct were also examined. The degree of displacement of the GFP from the plasma membrane was scored as follows: -, none; +, minimal; ++, moderate; +++, complete.

Suppression of Ras-Mediated Signaling by PAT Inhibitors

Ras-mediated cell proliferation involves activation of the Ras→Raf→mitogen activated/extracellular signal-regulated kinase→extracellular signal-regulated kinase pathway. To examine how the PAT inhibitors affect signaling through this pathway, the effects of compounds I to V on the phosphorylation of MEK in response to serum stimulation were examined (Fig. 7). Exposure of serum-starved MCF-7 or JC cells to serum resulted in a rapid increase in MEK phosphorylation. This increase in MEK phosphorylation was inhibited 40% to 90% by the PAT inhibitors as determined by quantitative image analysis. The total levels of MEK protein were not changed in the PAT inhibitor-treated cells, indicating that the effects of these compounds are due to decreased MEK phosphorylation rather than decreased expression of MEK protein.

In vivo Toxicity of PAT Inhibitors

Compounds I to V have been synthesized in amounts sufficient for preliminary characterizations of their toxicity and *in vivo* efficacies. Compounds I, II, III, and IV were soluble in DMSO/PBS (50:50) to at least 15 mg/mL (~30–50 mmol/L), whereas compound V was only soluble to 7 mg/mL (~23 mmol/L) in DMSO/PBS. Acute toxicity studies using i.p. dosing showed no immediate or delayed toxicity in female Swiss-Webster mice treated with up to at least 25 mg/kg of the compounds I, II, III, and IV. Repeated injections in the same mice every day over 15 days showed similar lack of toxicity. However, compound V administered i.p. at 25 mg/kg caused moderate toxicity, as evidenced by loss of body weight and poor coat appearance. Dosing at 12.5 mg/kg on the same schedule eliminated this effect.

In vivo Antitumor Activity of PAT Inhibitors

The *in vivo* antitumor activities of the PAT inhibitors were tested in a syngeneic tumor model that uses a transformed murine mammary adenocarcinoma (JC) cells growing in BALB/c mice (39). Female BALB/c mice were injected s.c. with 10⁶ JC cells suspended in sterile PBS. After palpable tumor growth, groups of mice were treated by i.p. injection with 30 µL of 50% DMSO (control) or a PAT inhibitor in 50% DMSO at a final dose of 25 mg/kg for 5 d/wk for a total of 10 doses. As indicated in Fig. 8,

compounds III and IV caused significant ($P < 0.05$) reductions in tumor growth on days 5 and 8. By day 15, all of the PAT inhibitors reduced tumor growth by 33% to 46%; however, the variability of the control group resulted in loss of statistical significance. These results indicate that PAT inhibitors can be chronically administered, resulting in inhibition of tumor growth.

Discussion

In recent years, the key roles of palmitoylated proteins in a number of cancer types have become increasingly clear. Oncogenes, such as H-, N-, and K-RAS2A, and the Src family of tyrosine kinases require posttranslational processing to function in cells (40–42). In fact, these proteins are nonfunctional if their sites of palmitoylation are removed, even if the proteins are constitutively activated by mutation (43). *Ras* genes are mutated in a high percentage of thyroid tumors, colon tumors, liver tumors, and pancreatic tumors, making these oncoproteins significant inducers of human cancer (reviewed in ref. 44). In addition, overexpression of normal Ras proteins leads to unregulated cell proliferation, and the fact that these

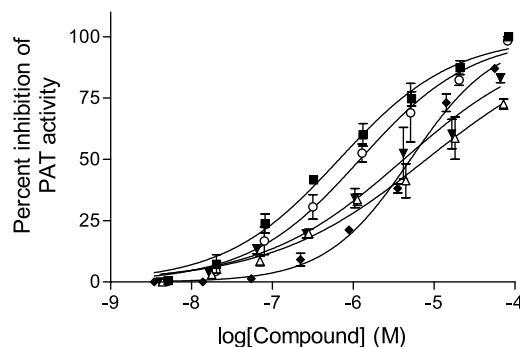
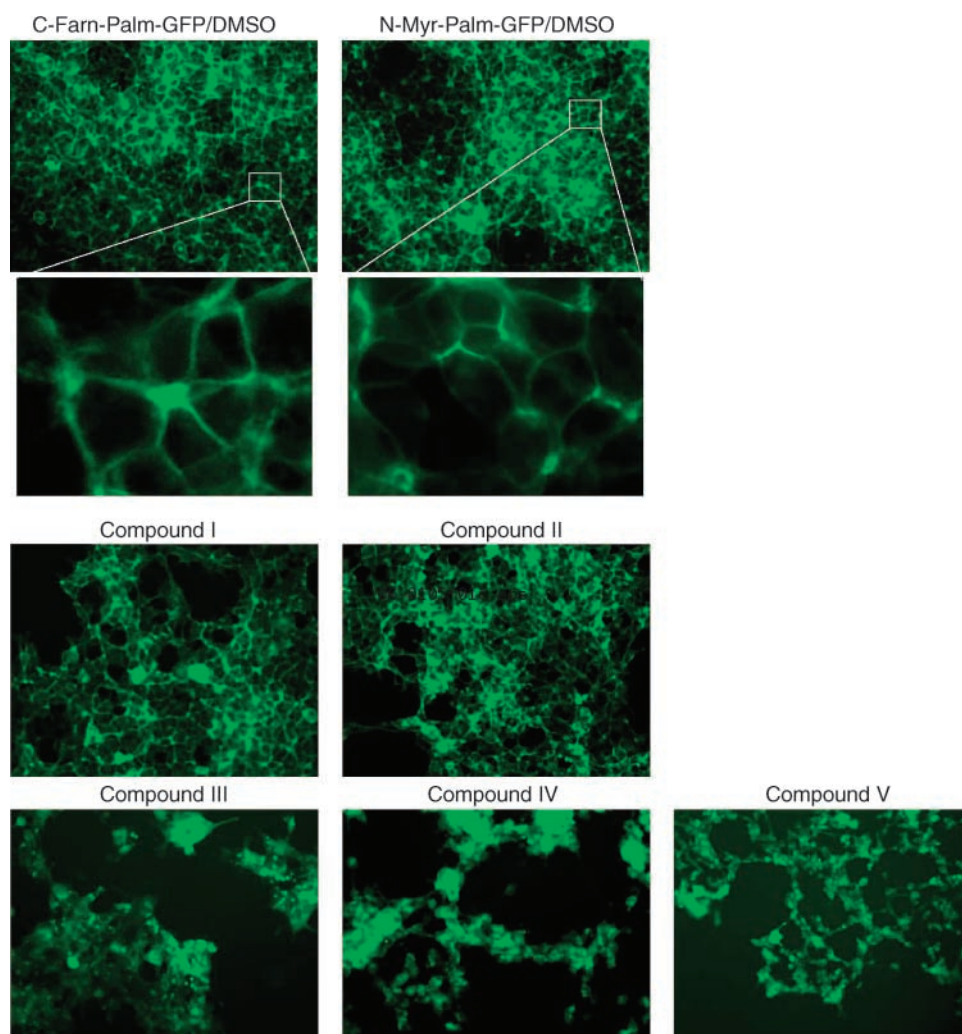


Figure 5. Dose-response curves for inhibition of PAT activity *in vitro*. *In vitro* palmitoylation assays were conducted in the presence of the indicated concentrations of compound I (△), compound II (▲), compound III (◆), compound IV (○), or compound V (■). Assays with compounds I to IV used the (NBD)-CLC(OMe)-Farn peptide and assays with compound V used the Myr-GC(NBD) peptide. Points, mean of triplicate samples; bars, SD.

Figure 6. Effects of PAT inhibitors on the subcellular localization of palmitoylated GFPs. 293 Tet-on cells, stably transfected with C-farn-palm-GFP or N-myr-palm-GFP expression vectors under the control of the tet-operator, were grown in 24-well plates. Cells were treated with 25 $\mu\text{g}/\text{mL}$ of the indicated compound for 0.5 h and induced with 1 $\mu\text{g}/\text{mL}$ doxycycline. The localization of the GFP fusion construct was visualized by fluorescence microscopy 24 to 48 h postinduction. Compounds I to IV are shown in C-farn-palm-GFP cell and compound V is shown in N-myr-palm-GFP cells. All images were taken with a $\times 40$ objective. Control images are digitally magnified.



proteins are downstream from growth factor receptors that may be inappropriately activated in many cancers. This makes it likely that Ras inhibitors will be therapeutically effective even in cancers in which mutation of *ras* genes is infrequent. In breast cancer, for example, <5% of cases report a *ras* mutation; however, there is considerable evidence to show that aberrant Ras activation and signaling promote breast cancer development (45). This hyperactivation of Ras is induced, at least in part, by constitutive activation of HER2, which occurs in $\sim 25\%$ of human breast cancers (46, 47). Persistent HER2 signaling-mediated cellular transformation is dependent on activation of H-Ras (48, 49). Similarly, Src-related tyrosine kinases are also frequently involved in promoting aberrant growth. For example, Lyn kinase activity is the predominant cellular Src kinase activity in glioblastoma cells (50).

Because the posttranslational processing of these proteins is critical for their function, the enzymes that carry out those reactions have been considered as prime targets for anticancer drugs. To date, the farnesyltransferases have

been the main focus for the development of anti-Ras agents (51, 52), whereas experimental drugs that inhibit the Src family have focused on inhibitors of their kinase activity (53, 54). With recent advancements in the identification and characterization of yeast and human PAT enzymes, it is now possible to begin to exploit these critical enzymes to develop new cancer therapies.

The identification and characterization of the yeast PAT enzymes *AKR1* and *ERF2/ERF4* have provided critical tools to begin the search for human homologues of these important enzymes (27, 28). The Davis laboratory has shown that the yeast protein Akr1p localizes to the Golgi (28) and contains ankryn repeats and a DHHC cysteine-rich domain (CRD). The ~ 50 -residue DHHC-CRD is a variant of the C2H2 zinc finger domain (55) and is defined by the core Asp-His-His-Cys tetrapeptide sequence. Homology searches have identified numerous proteins containing a DHHC sequence in *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Mus musculus*, *Homo sapiens*, and *Arabidopsis thaliana*, with at least 23 DHHC-containing proteins in humans (28).

Table 3. Potencies of PAT inhibitors toward human adherent tumor cell lines

Cell line	Tissue	Compound				
		I	II	III	IV	V
A498	Kidney	54 ± 32	84 ± 86	36 ± 10	94 ± 72	14 ± 1
Caco-2	Colon	6 ± 66	90 ± 77	38 ± 28	17 ± 0	6 ± 4
Du145	Prostate	90 ± 63	>145	32 ± 4	80 ± 10	6 ± 0
Panc-1	Pancreas	13 ± 42	>145	32 ± 4	29 ± 0	10 ± 2
SKOV3	Ovary	75 ± 37	>145	47 ± 15	101 ± 61	73 ± 101
HepG2	Liver	46 ± 36	>145	35 ± 1	56 ± 43	20 ± 12
T24	Bladder	23 ± 16	90 ± 77	30 ± 9	24 ± 28	7 ± 2
MDA-MB-231	Breast, ER–	60 ± 42	22 ± 20	32 ± 16	58 ± 41	9 ± 0
MCF7	Breast, ER+	3 ± 0	3 ± 0	10 ± 2	7 ± 3	2 ± 1

NOTE: Sparsely plated tumor cells were treated with the indicated PAT inhibitor for 48 hours, and cell number was determined using sulforhodamine B staining and compared with vehicle (DMSO)-treated cells. Values are the IC₅₀ (μmol/L) for at least three separate experiments. Abbreviations: ER–, estrogen receptor negative; ER+, estrogen receptor positive.

Although it is not likely that all proteins that contain a DHHC sequence are bona fide PATs, these studies have provided a reference point to begin the search for human PATs.

In the past year, several publications have shown that at least five DHHC-CRD proteins in humans have PAT

activity. Our group was the first to show that the huntingtin interacting protein 14 (HIP14/DHHC17) is a human PAT with specificity for the farnesyl palmitoylation motif found in COOH terminus of H-Ras and N-Ras (26). We also reported that this PAT has the ability to transform cells (26). Specifically, NIH/3T3 cells overexpressing HIP14

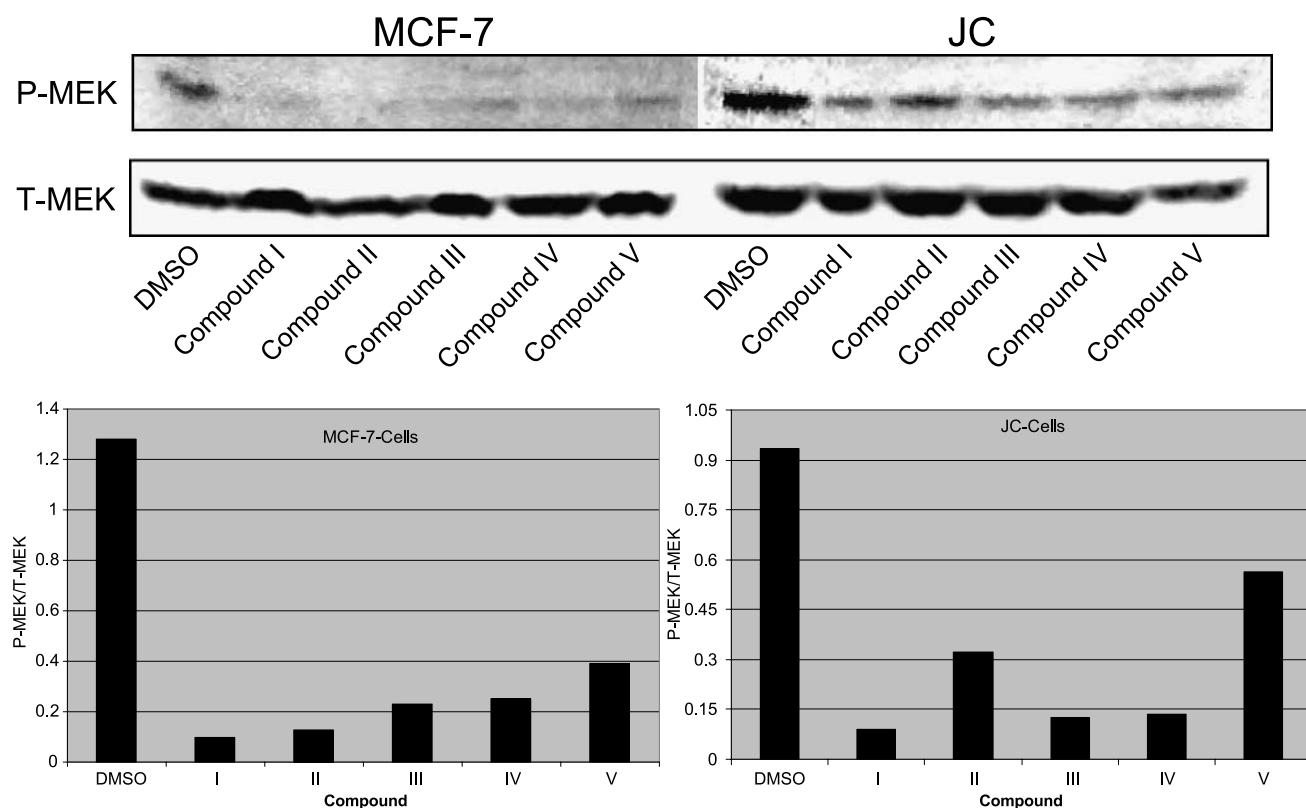


Figure 7. Suppression of signaling through Ras by PAT inhibitors. MCF-7 or JC cells were serum-starved and then treated with the indicated PAT inhibitor at a concentration of 3 to 4 μmol/L for 1 h before 1% fetal bovine serum was added. After 15 min, the cells were harvested, lysed, and total cell lysates were analyzed for levels of phospho-MEK (P-MEK) and total MEK (T-MEK) protein by Western blotting (top). Blots were imaged with a FUJIFILM Intelligent Dark Box II and quantified using Image Gauge 4.0 software. Bottom, ratios of P-MEK to T-MEK.

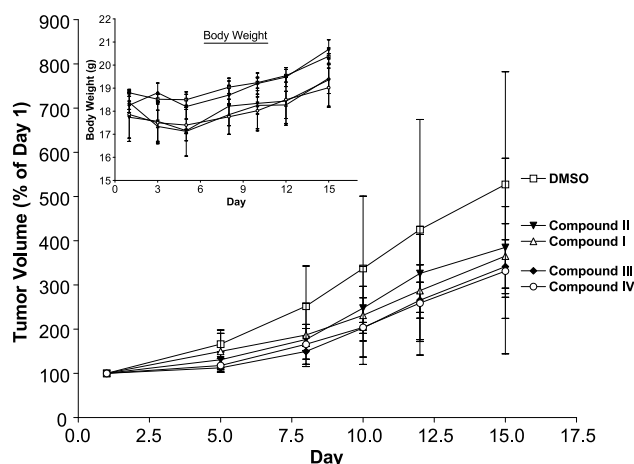


Figure 8. Antitumor activity of PAT inhibitors. BALB/c female mice were injected s.c. with JC cells suspended in PBS. After palpable tumor growth, animals were treated by i.p. injection of either 50% DMSO (control, \square) or 25 mg/kg compound I (\triangle), compound II (\blacktriangledown), compound III (\blacklozenge), or compound IV (\circ) for 5 d/wk for 2 wks for a total of 10 injections. Whole body weights (*inset*) and tumor volumes (*main panel*) on the indicated day after initiation of the treatments. *Points*, mean for each group ($n = 5$); *bars*, SD. $P < 0.05$ for compounds I and II at day 5.

formed foci on plastic and colonies in soft agar and aggressively grew as tumors in mice. Using these tumor-bearing mice, we showed the potential efficacy of small-molecule inhibitors of PATs as anticancer therapeutics by inhibiting tumor growth with a small interfering RNA that ablates HIP14 (26).

We now show that HIP14 is overexpressed in a number of human cancers, including colon, stomach, prostate, lung, and breast. Furthermore, we have shown that the increase in expression may correlate with increased severity of the disease. These results suggest that HIP14 may be a good pharmacodynamic marker for these diseases, as well as a viable target for their treatment. It can also be noted that Ras activity is increased in many of these cancers.

Other DHHC-CRD proteins that have been recently characterized include a Golgi-specific zinc finger protein (GODZ/DHHC3) that is involved in membrane protein trafficking (56), the c-Abl-associated protein Abl-philin 2 (Aph2/DHHC16) that may be involved in estrogen receptor stress-induced apoptosis (57), the Sertoli cell DHHC protein SERZ- β (DHHC7) that seems to have a role in maintaining Sertoli cell-differentiated functions (58), and a homologue of the ERF2/ERF4 complex in yeast DHHC9/GCP16 that has been shown to palmitoylate N-Ras and H-Ras in cells (59).

The identification and characterization of these PAT enzymes provided the tools necessary to begin the development of small-molecule inhibitors of their activity. The current study describes the design and implementation of three small-molecule screens used to identify compounds that inhibit type 1 and/or type 2 PAT activity. We have screened a library of diverse synthetic compounds and identified low molecular weight, drug-like molecules that inhibit type 1 or type 2 PAT activity. Although yet too

few and diverse for pharmacophore mapping, several of these hits segregated into five chemotypes and representatives of each of these chemotypes, i.e., compounds I to V, have been characterized further.

We have shown that compounds I to IV have selectivity for the type 1 palmitoylation motif and that compound V has selectivity for the type 2 palmitoylation motif. The specificity of the compounds suggests that they are peptide substrate competitors rather than palmitoyl-CoA competitors. This specificity is highly desirable as it will help limit the off-target effects of the PAT inhibitors when used as therapies. Importantly, because the compounds likely target the peptide binding site, they should not disrupt fatty acid biosynthetic pathways that also use palmitoyl-CoA. In addition to their use as leads for clinical development, these compounds are also useful probes for exploring the roles of palmitoylation in cancer biology. As our medicinal chemistry efforts proceed, we will be most interested in compounds that maintain this high level of selectivity.

Compounds I to V have IC_{50} values in the low $\mu\text{mol/L}$ range for inhibition of human tumor cell line proliferation. This is at least as good as that of 2-bromopalmitate and a cerulenin analogue designated as 16C (because it contains a saturated 16-carbon side chain designed to mimic palmitate; ref. 60), which are 4 and 1.3 $\mu\text{mol/L}$, respectively (25). More importantly, compounds I to V are small and hydrophilic compared with the fatty acid mimetics. These properties of the new PAT inhibitors should decrease nonselective binding and enhance the solubility of the compounds allowing for better formulations. Furthermore, because compounds I to V are screening hits, there is a great deal of chemical space available to explore in their optimization.

As part of the confirmation process, we have shown that all five of the PAT inhibitors function in intact cells to prevent palmitoylation-targeted GFP constructs from being localized to the plasma membrane. These results verify that the compounds can permeate cells and inhibit native PAT enzymes. In conjunction with this assay, we have shown by Western blotting that compounds I to IV, and to a lesser extent compound V, inhibit the Ras/Raf/Mek signaling cascade. Taken together, these experiments suggest that the PAT inhibitors attenuate Ras signaling by blocking its proper subcellular localization. Unlike the farnesyltransferase inhibitors there does not seem to be an escape mechanism for loss of palmitoylation.

To test whether any of the five PAT inhibitors could serve as chemotherapy agents, the compounds were administered to healthy mice to determine the maximum tolerated dose. Having established that mice could tolerate a reasonable quantity of each compound, tumor-bearing mice were dosed 10 times over the course of 2 weeks. The resulting delay in tumor growth was encouraging because these compounds are still only screening hits that have yet to be optimized. There is also the need to optimize the dosing and schedule of delivery of the compounds as their chemical properties are improved.

In conclusion, there is increasing evidence that palmitoylated proteins, and PATs themselves, have the ability to drive the progression of numerous cancer types. In particular, HIP14 expression is significantly elevated in a variety of solid tumors and may correlate with progression of the disease. We have identified several low molecular weight compounds that effectively inhibit HIP14 and/or other PATs. The pilot studies of *in vivo* antitumor activity indicate that these enzymes are viable targets for new therapeutics. Additional development of the PAT inhibitors through medicinal chemistry and absorption, distribution, metabolism, and elimination profiling will be critical in lead optimization. Analysis of their cellular and *in vivo* effects will also be crucial for evaluating the clinical potential of this new class of targeted compounds.

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