

## Probe Report

**Title:** Small-Molecule Inhibitors of Vaccinia-H1-Related Phosphatase VHR

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**Assigned Assay Grant #:** 1 R03 MH084230-01A1

**Screening Center Name & PI:** Burnham Center for Chemical Genomics & Dr John C Reed

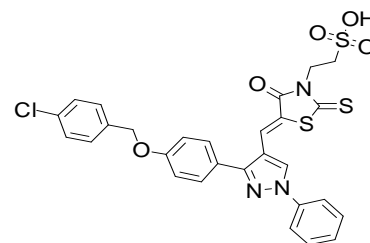
**Chemistry Center Name & PI:** Burnham Center for Chemical Genomics & Dr John C Reed

**Assay Submitter & Institution:** Lutz Tautz & Burnham Institute for Medical Research

**PubChem Summary Bioassay Identifier (AID):** 1661

**Probe Structure & Characteristics:** CID-6161281

(Cmpd internal # is MLS-0425632)



**ML113**

CID/ML	Target Name	IC50/EC50 (nM) [SID, AID]	Anti-target Name(s)	IC50/EC50 (uM) [SID, AID]	Selectivity	Secondary Assay(s) Name: IC50/EC50 (nM) [SID, AID]
6161281 ML113	VHR	IC50 18 (nM) SID 85256223 AID 2074	MKP-1 in-class dual specific PTP	IC50 0.46 (uM) SID 85256223 AID 2083	25.6X	N/A
			HePTP out-of-class classical PTP	IC50 0.62 (uM) SID85256223 AID 2082	34.4X	

### Recommendations for the scientific use of this probe:

Loss of VHR phosphatase causes cell cycle arrest in HeLa carcinoma cells, suggesting that VHR inhibition may be a useful approach to halt the growth of cancer cells. VHR is also upregulated in several cervix cancer cell lines as well as in carcinomas of the uterine cervix. Here we report the development of multidentate small-molecule inhibitors of VHR that inhibit its enzymatic activity at nanomolar concentrations and are selective for VHR over HePTP and MKP-1.

This novel chemical probe appears to interact with both the phosphate-binding pocket and several distinct hydrophobic regions within VHR's active site, so it will serve as a useful tool

in probing these interactions and elucidating the molecular mechanism of selectivity against this phosphatase and the functional consequences for cancer biology.

The probe decreased the proliferation of cervix cancer cells at low micromolar concentrations, while growth of primary normal keratinocytes was not affected. Thus this probe may also be a starting point to develop drugs for the treatment of cervical cancer.

## 1. Scientific Rationale for Project

### Specific aims

The overall goal is to develop selective and efficient VHR inhibitors for basic research on signal transduction processes and MAP kinase regulation. For the future, this work could also create proof-of concept evidence that VHR is a good drug target for the treatment of cervical cancer. Some of these inhibitors may turn out to be suitable for further development towards clinical use.

### Background and Significance

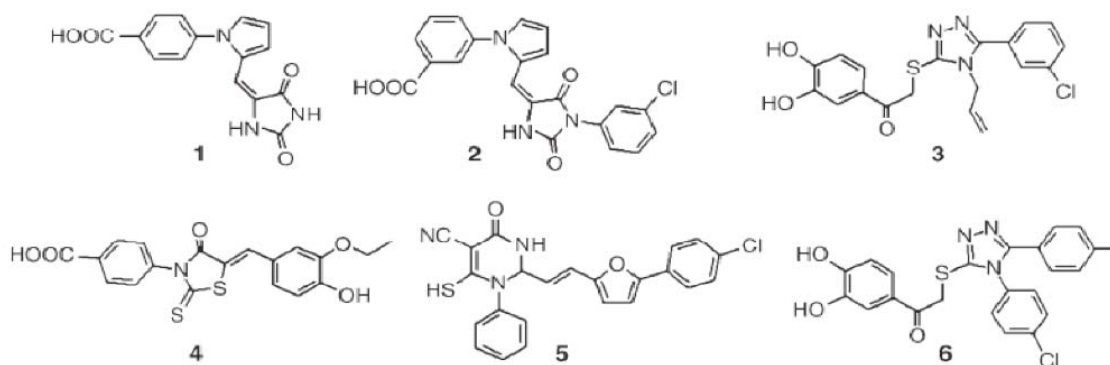
The Vaccinia H1-related (VHR) protein tyrosine phosphatase is a relatively small member of the subclass of dual-specificity phosphatases<sup>1</sup> with only 185 amino acids (mw 21 kDa) and with no apparent targeting domain or docking site.<sup>2</sup> Compared to the phospho-tyrosine (pTyr)- specific classical PTPs, the crystal structure of VHR revealed a much shallower active site, which allows VHR to act on both pTyr and phospho-threonine (pThr) in its substrates.<sup>3</sup> VHR dephosphorylates and thereby inactivates extracellular signal-regulated kinases Erk1/2 and c-Jun N-terminal kinases Jnk1/2, but not p38.<sup>4-6</sup> These mitogen-activated protein kinases (MAP kinases) mediate major signaling pathways triggered by extracellular growth factor, stress, or cytokines<sup>7</sup> and regulate cellular processes such as differentiation, proliferation and apoptosis.<sup>8,9</sup> Unlike many MKPs, VHR expression is not induced in response to activation of MAP kinases<sup>10</sup> but is instead regulated during cell cycle progression.<sup>11</sup> The loss of VHR causes cell cycle arrest in HeLa carcinoma cells, suggesting that VHR inhibition may be a useful approach to halt the growth of cancer cells without the detrimental effects on normal cells. The assay provider recently reported that VHR is upregulated in several cervical cancer cell lines as well as in squamous intraepithelial lesions and squamous cell carcinomas of the uterine cervix.<sup>12</sup>

## 2. Project Description

### a. Describe the original goal for probe characteristics as identified in the CPDP.

The ideal probe would be a new scaffold of improved potency (< 2-3  $\mu$ M) over current probes in the literature *and* improved selectivity towards other phosphatases (at least 2-fold over either MKP-1 or HePTP). However, as there are very few potent phosphatase inhibitors and even fewer selective ones, any improvement in either potency or selectivity would be useful. Additionally activity in a cell based assay for direct substrates is a desired characteristic; however, if none of the scaffolds obtained by catalog or by medicinal chemistry efforts have cell activity, the probes will still be useful as *in vitro* biochemical tools.

In the original CPDP, several compounds are disclosed as prior art, but none are more than potent than  $\sim 4$   $\mu$ M, reported as selective for VHR1 nor cell active without cellular toxicity and are reiterated below from H. Park *et al.* "Discovery of VHR Phosphatase Inhibitors with Micromolar Activity based on Structure-Based Virtual Screening" (2008) *J Med Chem* **3**:877 – 880 (*the authors do not specify the stereochemistry as E or Z for these olefins. However, they do mention that the vendor is Interbioscreen*).



**Figure 1.** Chemical structures of prior art VHR inhibitors from Park *et al.* 2008

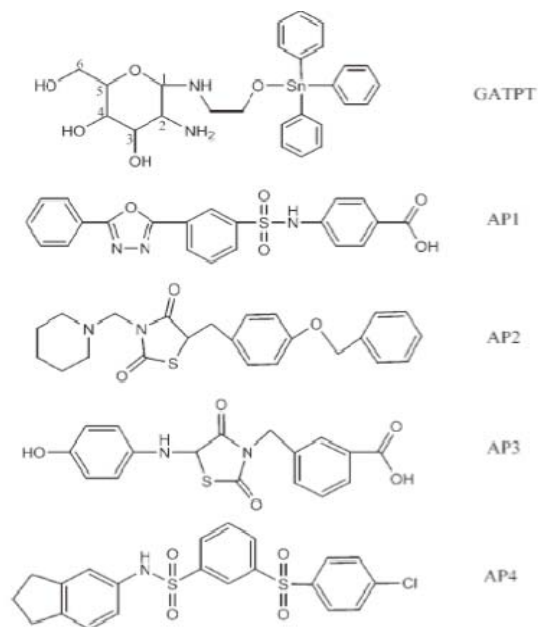
There was no cell activity reported for compounds **1 – 6** in **Figure 1, Table 1** (but they may have not been tested in cell assays). Compounds **1** and **2** were purchased and provided to the assay provider for cell activity testing and reference.

<b>Table 1.</b> The inhibitory activities of compounds 1 -6 against VHR <sup>a</sup> (adapted from Park <i>et al.</i> 2008)				
Compound	%Inhibition at 50 $\mu$ M	IC <sub>50</sub> ( $\mu$ M))	CID	SID(s)
1	82.8	3.7	2932059	34500079, 4450071
2	83.5	4.7	1210330	51674707, 29248963, 6159808
3	77.2	13.9		
4	72.1	20.0		
5	74.1	24.2		
6	70.9	46.5		
<sup>a</sup> At all concentrations of the inhibitors, the inhibition assay was performed in duplicates				

Additional VHR inhibitors (**Table 2, Figure 2**) were reported by Zhi *et al.* "Identification of a Potent Inhibitor of Human Dual-Specific Phosphatase, VHR, from Computer-Aided and NMR-Based Screening to Cellular Effects" (2007) *ChemBioChem* **8**:2092-2099 GATPT is a stannous (tin containing compound and toxic) of no chemical interest or tractability. Has some reported cell activity, however it has been shown to be toxic.

<b>Table 2.</b> Inhibitory activities of compounds against VHR <sup>a</sup> (adapted from ZHI <i>et al.</i> 2007)		
Compound	%Inhibition at 100 $\mu$ M	IC <sub>50</sub> ( $\mu$ M))
GATPT	95 $\pm$ 2	2.92
AP1	67 $\pm$ 3	56.95
AP2	74 $\pm$ 2	100.51
AP3	63 $\pm$ 2	45.84
AP4	10 $\pm$ 2 <sup>b</sup>	26,200
<sup>b</sup> Compound concentration was 10 mM		

The following publications refer to these existing probes with PMIDs: 7556642, 11563920, 11755399, 17933004, and 18236492.



**Figure 2.** Chemical structures of VHR inhibitors from Shi *et al.* 2007

- b. For each assay implemented and screening run please provide:
- i. PubChem Bioassay Name(s), AID(s), Assay-Type (Primary, DR, Counterscreen, Secondary)

Table 3. PubChem Assay Summaries for Probe project					
PubChem BioAssay Name	AIDs	Probe Type	Assay Type	Assay Format	Assay Detection & well format
VHR1 Inhibitors – Primary	1992	Inhibitor	Primary	Biochemical	Absorbance 96-well
VHR1 – Confirmatory (CP & DR)	1992, 2004	Inhibitor	Confirmatory	Biochemical	Absorbance 96-well
VHR1 Inhibition – SAR support	2074	Inhibitor	Secondary	Biochemical	Fluorescence 96-well
HePTP Inhibition - SAR	2082	Inhibitor	Counterscreen	Biochemical	Fluorescence 96-well
MKP-1 Inhibition– SAR	2083	Inhibitor	Counterscreen	Biochemical	Fluorescence 96-wells

- ii. Assay Rationale & Description (when describing primary screen it would be useful to see standard metrics like, Z', S:B for the optimized assay).

The Vaccinia H1-related (VHR) PTP is a dual-specific Erk and Jnk phosphatase. This primary screen (AID1992) is based on a biochemical assay that employs a colorimetric readout based on the enzyme's ability to liberate phosphate from *para*-nitrophenyl phosphate (pNPP) and the reaction or the released phosphate with Biomol Green reagent.<sup>13</sup> Following initial screening the hits are confirmed for activity using the same assay but from re-ordered stock solutions in duplicate single concentrations (AID1992), and then to determine the potency from full dose response curves (AID2004).

#### Table of reagents and source.

##### Assay materials:

Table 4. Reagents used for the HTS experiments	
Reagents	Supplier/Vendor
Recombinant VHR (expressed & purified as per ref. 14)	Dr. Tautz's Lab
p-Nitrophenyl phosphate (pNPP)	Sigma (St. Louis, MO)
BIOMOL GREEN	BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA)
Bis-Tris pH 6.0 reaction buffer	Sigma (St. Louis, MO)
Dithiothreitol (DTT)	Sigma (St. Louis, MO)

##### VHR materials:

- 1) Recombinant VHR was provided by Prof. Lutz Tautz (Burnham Institute for Medical Research, San Diego, CA)
- 2) Assay buffer: 0.1 M Bis-Tris pH 6.0 with 1 mM dithiothreitol (DTT)
- 3) 4 mM pNPP
- 4) BIOMOL Green 2:1 addition to quench a 70 uL reaction volume

##### VHR protocol:

Each reaction contained 180 nM VHR, 4 mM pNPP, and 0.02 mg/mL compound in 0.1 M Bis-Tris pH 6.0 reaction buffer with 1 mM dithiothreitol (DTT) present. The final volume amounted to 70 µL and contained 1.4% DMSO. The reaction was initiated by addition of pNPP after a preincubation of the enzyme with compounds for 10 min at room temperature. After 15 min, the reaction was quenched by addition of 140 µL BIOMOL GREEN reagent, and the pNPP hydrolysis was determined by measuring the absorbance of the complexed free phosphate at 620 nm. The nonenzymatic hydrolysis of the substrate was corrected by measuring the negative control without addition of enzyme. Other controls included a positive control (no inhibitor added), a background control (no substrate added), and a control with 200 uM of the general PTP inhibitor sodium orthovanadate. To quantitate the inhibitory efficacy of the library compounds, we determined the ratio of inhibition in

comparison to the positive control. Every compound with >60% inhibition was cherry-picked and rescreened to confirm it as a hit (AID1992) and confirmed for initial potency (AID2004).

#### **Rationale for confirmatory, counter and selectivity assays:**

**VHR SAR assay:** A higher sensitivity fluorogenic assay was developed and performed to study the structure-activity relationship on analogs of the confirmed VHR hits reported in AID 2004. This VHR biochemical assay (AID2074) employs a fluorescent readout based on the enzyme's ability to catalyze the hydrolysis of 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) in the presence of an inhibitor.

**Counterassay (MKP-1):** MAP kinase phosphatase 1 (MKP-1) is, like VHR, a dual-specific phosphatase and structurally closely related to VHR. MKP-1 also shares a physiological substrate with VHR, namely the extracellular signal-regulated kinases Erk1/2. Therefore, MKP-1 was chosen to assess the selectivity of the VHR1 inhibitors reported in AID 2004. This biochemical assay for MKP-1 (AID2083) employs the same fluorescent readout as the VHR SAR assay.

**Selectivity Assay (HePTP):** In contrast, the hematopoietic tyrosine phosphatase (HePTP) is a *classical* pTyr-specific phosphatase. HePTP shares a physiological substrate with VHR, namely the extracellular signal-regulated kinases Erk1/2, and therefore was chosen to assess the selectivity of the VHR1 inhibitors reported in AID 2004. This biochemical assay for HePTP (AID2082) also employs the same fluorescent readout as the VHR SAR assay. The commonality of the assay format and readout ensure comparability of potency values in these three assays.

**General assay protocol:** The PTP-catalyzed hydrolysis of 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) in the presence of compound was assayed at 30°C in a 60 uL 96-well format reaction system in 150 mM Bis-Tris, pH 6.0, assay buffer having an ionic strength of 150 mM (adjusted with NaCl) and containing 1 mM DTT and 5%DMSO. At various concentrations of the compound, the initial rate at fixed DiFMUP concentration (equal to the corresponding  $K_m$  value for each PTP) was determined using a FLx800 microplate reader (Bio-Tek Instruments, Inc.), an excitation wavelength of 360 nm, and measuring the emission of the fluorescent reaction product 6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU) at 460 nm. The nonenzymatic hydrolysis of the substrate was corrected by measuring the control without addition of enzyme. The  $IC_{50}$  value was determined by plotting the relative activity versus inhibitor concentration and fitting to eq 1 using the software GraphPad Prism (GraphPad Software, Inc.).

$$V_i/V_0 = IC_{50}/(IC_{50} + [I]) \quad (\text{eq. 1})$$

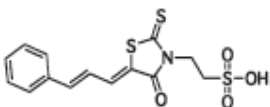
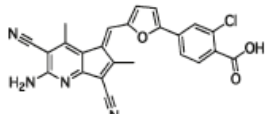
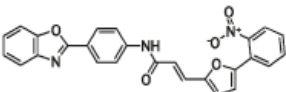
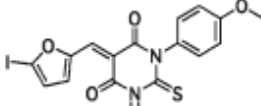
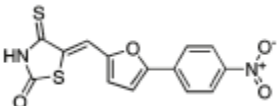
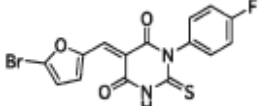
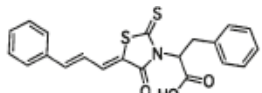
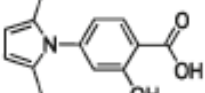
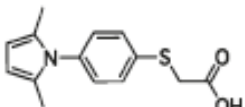
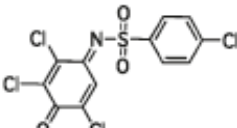
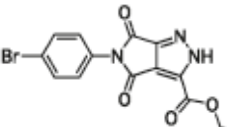
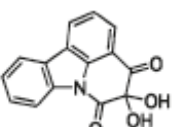
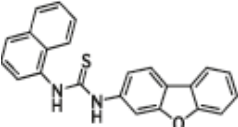
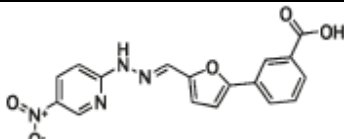
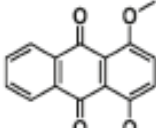
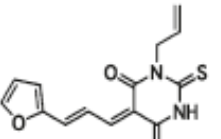
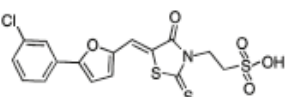
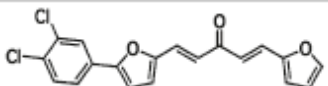
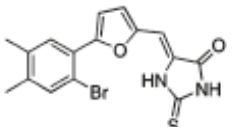
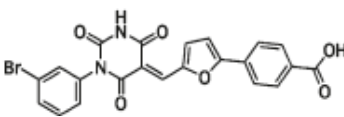
In this case,  $V_i$  is the reaction velocity when the inhibitor concentration is  $[I]$ ,  $V_0$  is the reaction velocity with no inhibitor, and  $IC_{50} = K_i + K_i[S]/K_m$ .

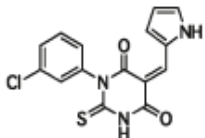
#### **iii. Center Summary of Results**

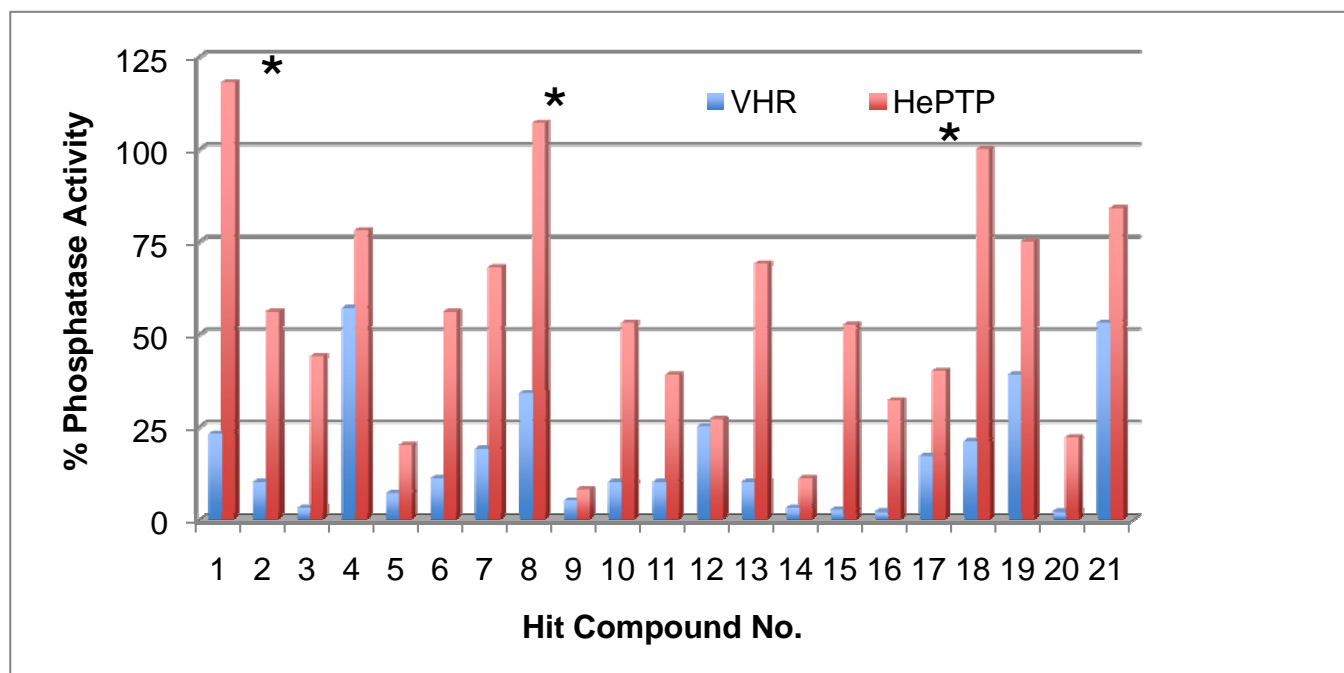
We have completed the primary screen of 291,018 compounds from the MLSMR library (AID1654) using a colorimetric assay that measures the enzyme's ability to liberate phosphate from *para*-nitrophenyl phosphate (PNPP) and detection of the released phosphate with Biomol Green reagent. However, at this juncture no VHR compounds of <2-3 uM potency with selectivity against other phosphatases (e.g. HePTP) were found. This probe report describes the results of a parallel screen of the 49,840 drug-like molecules of the DIVERSet library from ChemBridge, Inc. (San Diego, CA), at the concentration of 0.02 mg/mL in a 96-well format using an *in vitro* colorimetric phosphatase assay, in collaboration with the assay provider. The average  $Z'$  for this assay was 0.85, the signal to background ratio was 3.8, the signal to noise ratio was 46.6 and the signal window was 34.7. We found that 221 compounds inhibited VHR's enzymatic activity to >60% (average of  $n=2$ ) compared to a no-inhibitor control. For further evaluation, a total of 56 compounds were picked that inhibited VHR>90% (average of  $n=2$ ). Michaelis-Menten kinetic studies revealed 21 hits that inhibited the enzyme with  $K_i$  values <20 uM (**Table 5**). Clustering the 21 compounds by a Tanimoto distance<sup>15</sup> of 0.5 resulted in 12 different clusters and singletons, respectively, indicating a quite diverse chemical space covered by these molecules. The

most active hit, 2-((*Z*)-4-oxo-5-((*E*)-3-phenylallylidene)-2-thioxothiazolidin-3-yl)ethane-sulfonic acid, SID17460170 (**1**), inhibited VHR with a  $K_i$  value of 0.81  $\mu$ M. In a counter screen against the protein tyrosine phosphatase, HePTP, only **1** and 1,4-dimethoxyanthracene-9,10-dione, SID17438068 (**8**), and (1*E*,4*E*)-1-[5-(3,4-dichlorophenyl)furan-2-yl]-5-(furan-2-yl)penta-1,4-dien-3-one, SID17461020 (**18**) were found to be selective for VHR (**Figure 3**).

**Table 5.** Most Potent VHR Screening Hits

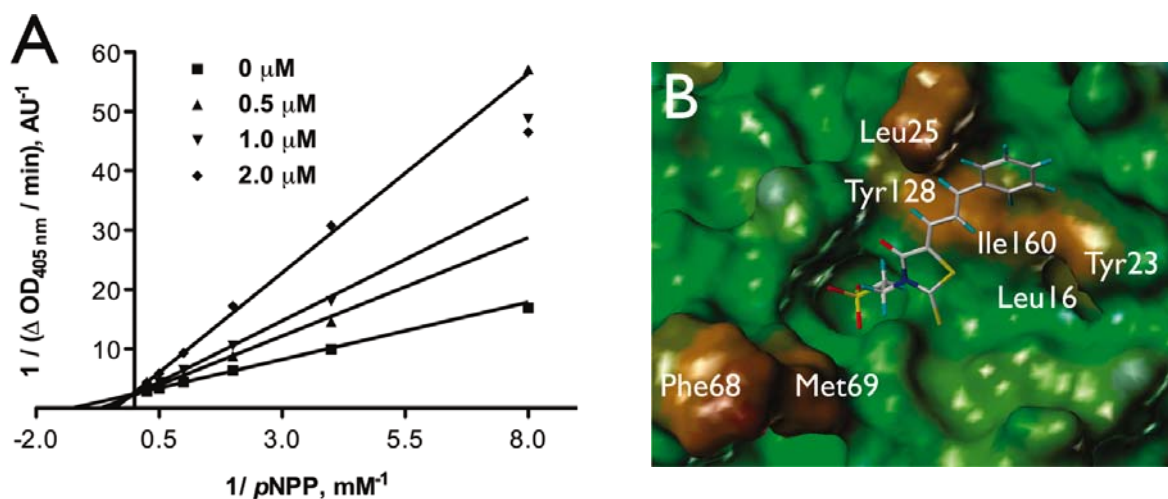
#	PubChem SID	Structure	$K_i$ ( $\mu$ M)	#	PubChem SID	Structure	$K_i$ ( $\mu$ M)
1	17460170		0.809	12	17468205		3.87
2	17467259		1.76	13	17462864		4.44
3	17459623		1.86	14	17474557		1.21
4	17470230		2.01	15	17465980		12.8
5	17464133		2.09	16	17437417		13.4
6	17486385		2.64	17	17444164		13.8
7	17462192		2.94	18	17461020		13.8
8	17438068		3.07	19	17464863		15.8
9	17484886		3.13	20	17460326		15.9
10	17464668		3.19	21	17466330		16.2

11	17457520		3.49				
K <sub>i</sub> value < 20 μM. Compounds <b>11</b> , <b>13</b> , <b>19</b> , and <b>21</b> are mixtures of <i>E/Z</i> -isomers.							



**Figure 3.** Profiling of the 21 most active VHR screening hits at 20 μM against VHR (AID 2074) & HePTP (AID 2082). Asterisks indicate compounds 1, 8, and 18 (**Table 5**), which appeared to be selectively inhibiting VHR without HePTP inhibition.

Of these three compounds, only SID17460170 (**1**), showed a clear competitive inhibition pattern as shown in the Lineweaver-Burk plot in **Figure 4A**.



**Figure 4.** Characterization of SID17460170 (**1**). (A) Michaelis-Menten kinetic measurements,

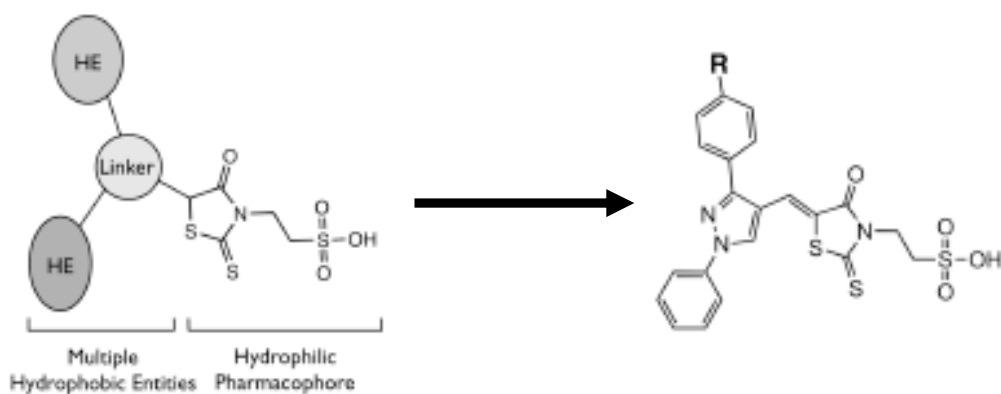
Lineweaver-Burk plots for SID17460170 (**1**) with VHR. (B) FlexX docking of SID17460170 (**1**) into the active site of VHR crystal structure (PDB code 1J4X). The color code of the MOLCAD surface represents the lipophilic potential (brown: most hydrophobic, blue: most hydrophilic).

### c. Probe Optimization

#### i. Describe SAR & chemistry strategy (including structure and data) that led to the probe.

**In Silico Docking.** To study the molecular basis for inhibition of VHR, we first used *in silico* docking to dock SID17460170 (**1**) and SID17438068 (**8**) into the active site of the VHR crystal structure (PDB code 1J4X, ref 16). Compound SID17438068 (**8**) failed to dock into the catalytic pocket, further supporting an inhibition mechanism other than competitive. Because quinones like SID17438068 (**8**) are known to deactivate PTPs by oxidizing the catalytic cysteine residue,<sup>7</sup> so we discarded SID17438068 (**8**) from further investigation. As for compound SID17460170 (**1**), the docking suggested that the sulfonic acid moiety functions as a phosphate mimic and binds into the catalytic pocket, forming a network of hydrogen bond interactions with the phosphate binding loop, also called P-loop (Figure 2B). In support of this, similar compounds lacking the sulfonic acid moiety did not inhibit VHR. Furthermore, the docking suggested that the oxo-thioxothiazolidine ring interacts with the rim of the catalytic pocket, whereas the diene linker and the phenyl ring make van der Waals interactions with a hydrophobic region, mainly formed by Leu25 and Tyr128 (Figure 2B, above).

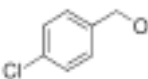
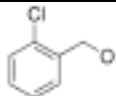
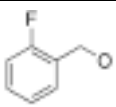
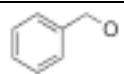
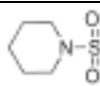
**SAR Analysis and Analogs of Lead Compound 1.** By further analyzing the lipophilic potential of VHR's active site surface area, we found a total of three distinct hydrophobic regions surrounding the catalytic pocket (Figure 2B). Compared to active site properties of solved PTP structures and homology models,<sup>17</sup> this feature seems to be rather unique to VHR and, therefore, could be exploited for designing selective inhibitors. The exposed hydrophobic patches are formed by Leu25/Tyr128 and Leu16/Tyr23, which make up large portions of the substrate binding site,<sup>10</sup> as well as by Phe68/ Met69, which is part of a loop opposite of the catalytic pocket (Figure 2B). In an attempt to find analogs of **1** that could target more than one of these hydrophobic regions, we kept the oxo-thioxothiazolidinyl-ethanesulfonic acid moiety as hydrophilic pharmacophore, and subjected it to a substructure search among commercially available compounds. The pharmacophore model and limited R-group search is summarized below:



Six structures were identified that fulfilled the requirement of having additional multiple hydrophobic entities to interact with multiple hydrophobic regions in VHR's active site. All compounds contained a 1-phenyl-substituted pyrazole ring, which at its 3-position linked to another, more variable entity (see **Table 6**). We obtained all six analogs and subjected them to direct measurements of VHR inhibition. Indeed, compounds CID-6161281, -6274486, -5932584, -5998084, and -6166563 (**SA1-5**) showed excellent IC<sub>50</sub> values of 18,

71, 74, 78, and 268 nM, respectively (**Table 6**). An aromatic group in position 'R', such as the benzoxy group in compounds CID-6161281, -6274486, -5932584, and -5998084 (**SA1-4**), seemed to be very well accommodated by the protein, whereas sterically more demanding structures, such as the piperidinesulfonyl group in CID-6166563 (**SA5**) were less favorable. On the other hand, groups such as methyl in CID-6001181 (**SA6**) seemed to be too small to provide additional binding energy through van der Waals interactions. Interestingly, compounds CID-6161281, -6274486, -5932584, and -5998084 (**SA1-4**) only differ in their substituents at the benzoxy group. Chlorine in the *para* position was most favorable CID-6161281 (**SA1**), whereas substituents in the *ortho*-position, such as chlorine CID-6274486 (**SA2**) or fluorine CID-5932584 (**SA3**), had no effect on inhibitory activity against VHR *in vitro*. Testing the five best inhibitors CID-6161281, -6274486, -5932584, -5998084, and -6166563 (**SA1-5**) against a panel of related PTPs demonstrated that they were at least 1 order of magnitude less potent for MKP-1 and HePTP (**Table 6**). MKP-1 and HePTP share the same physiological substrate with VHR, namely, the MAP kinase Erk, and MKP-1 is structurally closely related to VHR.

**Table 6.** *In vitro* potency and Selectivity of 1-phenylpyrazole VHR inhibitor analogs

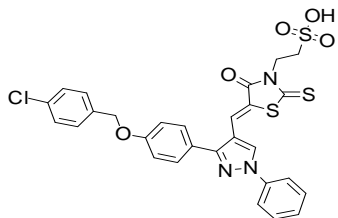
ID MLS- Internal No. (SA1#)	Structure R =	PubChem SID	PubChem CID	VHR IC <sub>50</sub> (uM) AID 2074	MKP-1 IC <sub>50</sub> (uM) AID 2083	HePTP IC <sub>50</sub> (uM) AID 2082
MLS-0425632 (SA1)		85256223	6161281	0.018	0.46	0.62
MLS-0425633 (SA2)		85256224	6274486	0.071	0.47	1.2
MLS-0425634 (SA3)		85256225	5932584	0.074	0.52	0.87
MLS-0425635 (SA4)		85256226	5998084	0.078	0.78	1.5
MLS-0425636 (SA5)		85256227	6166563	0.27	2.8	2.4
MLS-0425637 (SA6)	CH <sub>3</sub>	85256228	6001181	3.1	ND	ND

### 3. Probe

#### a. Chemical name of probe compound

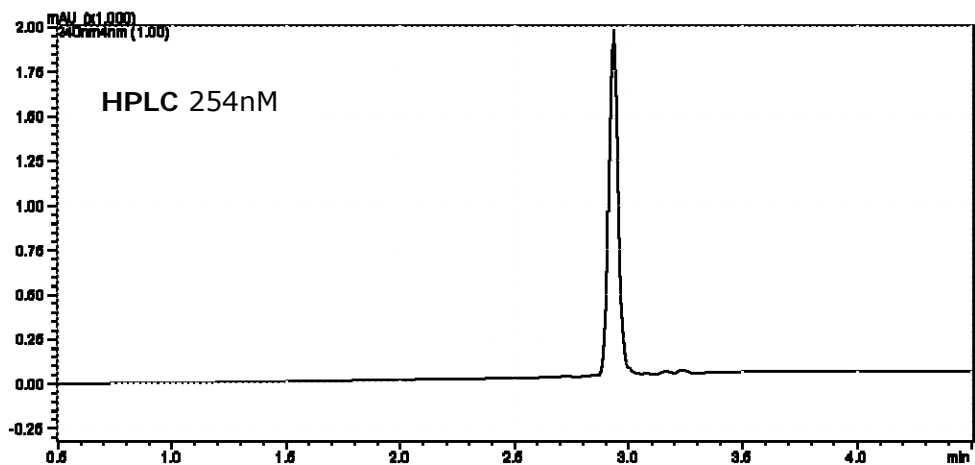
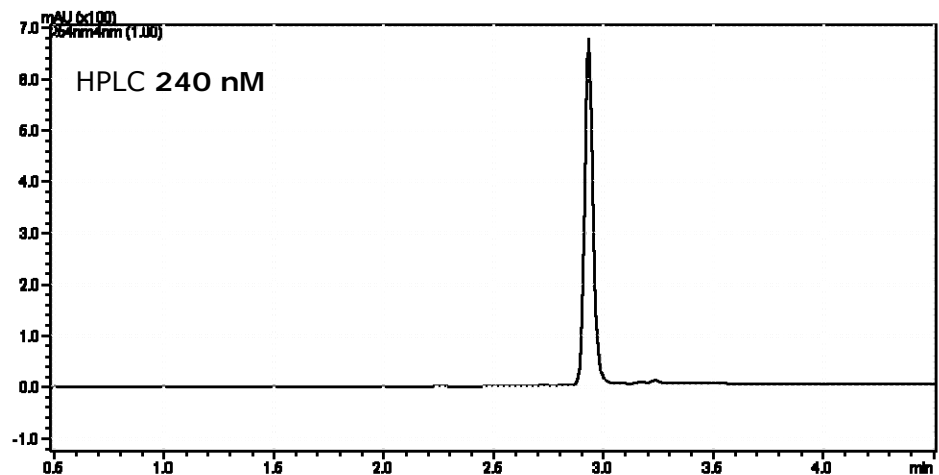
**IUPAC:** 2-[(5Z)-5-[[3-[4-[(4-chlorophenyl)methoxy]phenyl]-1-phenylpyrazol-4-yl]methylidene]-4-oxo-2-sulfanylidene-1,3-thiazolidin-3-yl]ethanesulfonic acid  
[ML113]

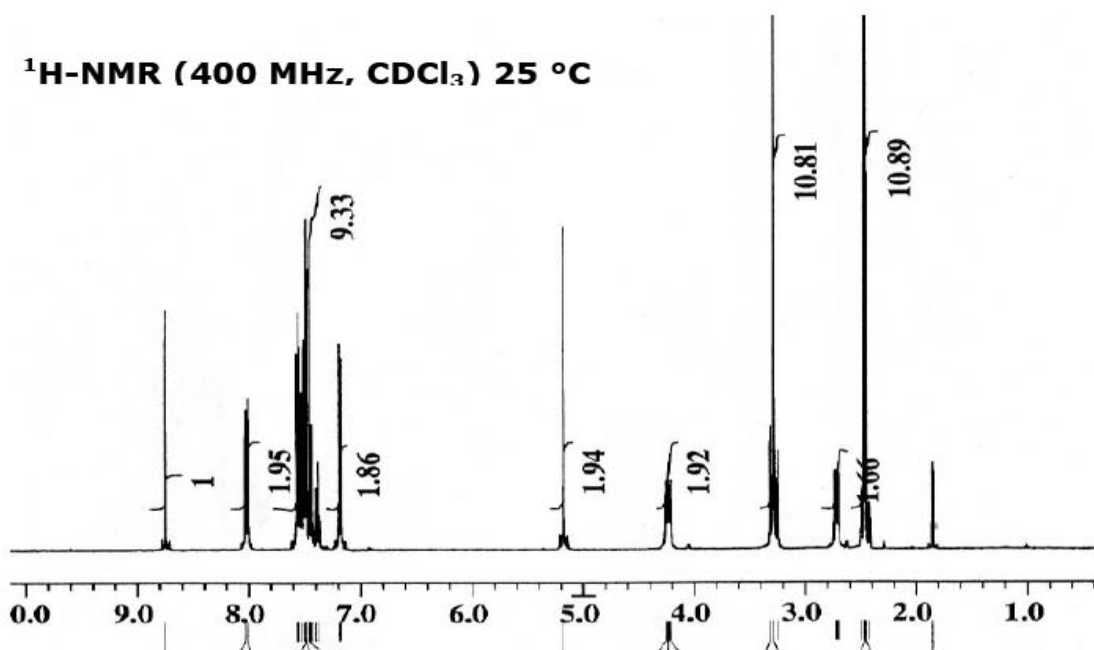
**b. Probe chemical structure including stereochemistry if known**



**c. Structural verification information of probe SID**

Probe SID number is SID 85256223 Purity: >95% (HPLC-MS)





**d. PubChem CID (corresponding to the SID)**

PubChem CID is 6161281 (corresponding to SID 85256223)

**e. If available from a vendor, please provide details**

This probe molecule is available from Princeton BioMolecular Research, Inc.

(Monmouth Jct, NJ) with a catalog number of OSSK\_022127 ([www.princetonbio.com](http://www.princetonbio.com))

**f. Provide MLS# that verifies the submission of probe molecule and related samples that were submitted to the SMR collection:**

<b>Table 7.</b> Submission of Probe and 5 analogs to the MLSMR							
Probe/ Analog	MLS# (DPI)	MLS-# (BCCG#)	SID	CID	Source (vendor or BCCG syn)	Amt (mg)	Date submitted
Probe ML113	MLS002554749	0425632	85256223	6161281	PrincetonBio	50	10/16/09
Analog1	MLS002554750	0425633	85256224	6274486	PrincetonBio	20	10/16/09
Analog2	MLS002554751	0425634	85256225	5932584	PrincetonBio	20	10/16/09
Analog3	MLS002554752	0425635	85256226	5998084	PrincetonBio	20	10/16/09
Analog4	MLS002554753	0425636	85256227	6166563	PrincetonBio	20	10/16/09
Analog5	MLS002554754	0425637	85256228	6001181	PrincetonBio	20	10/16/09

**g. Describe mode of action for biological activity of probe**

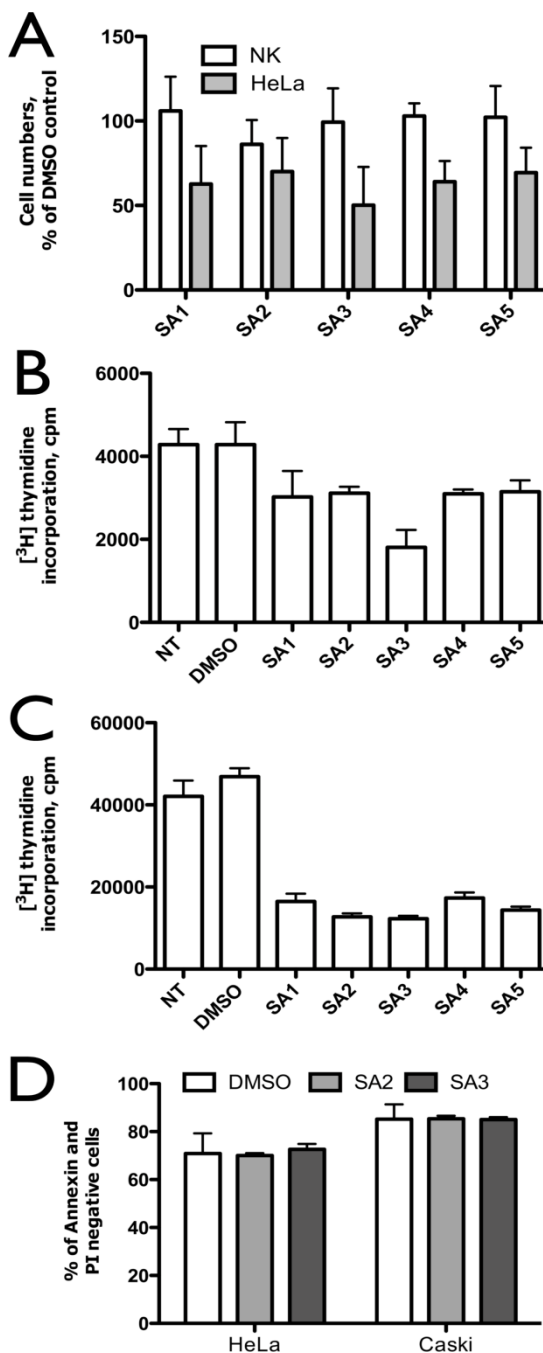
The resulting lead compound SID17460170 (**1**) competitively inhibited VHR with a K<sub>i</sub> value of 0.81 uM and exhibited a promising degree of selectivity for VHR among other PTPs. *In silico* docking of SID17460170 (**1**) into the active site of VHR and SAR analysis suggested a binding mode, in which the sulfonic acid moiety of SID17460170 (**1**) binds through a network of hydrogen bond interactions into the phosphate binding pocket, whereas the thiazolidine heterocycle interacts with the rim of the pocket and the phenyl-allyl moiety makes van der Waals interactions with side chains of hydrophobic amino acids that flank the

active site. Besides highly improved potency against VHR with  $IC_{50}$  values as low as 18 nM, these multidentate inhibitors were at least 1 order of magnitude less potent for any other PTP tested, including HePTP and MKP-1, which share the same physiological substrate with VHR. This result provides a good example for a general applicable concept, in which targeting unique surface features outside of the catalytic pocket can generate selective small-molecule inhibitors for individual members of the PTP family.<sup>18</sup>

The assay provider, Dr. Tautz found that VHR inhibitors are able to pass cell membrane barriers and target VHR in cultured cells.<sup>18</sup> In particular, he tested the compounds in cervical cancer cells, which were shown earlier to express higher levels of endogenous VHR compared to noncancerous cells of the cervix. Indeed, incubation of the cancer cell line HeLa and CaSki with the inhibitors at 20  $\mu$ M induced a very significant inhibition of cell proliferation after an incubation period as short as 24 h.

It is interesting to note that CID-5932584 (**SA3**) was more effective in inhibiting proliferation than CID-6161281 (**SA1**), although its  $IC_{50}$  value is four times higher *in vitro*. CID-5932584 (**SA3**) also exhibited greater antiproliferative effects than CID-6274486 (**SA2**) and CID-5998084 (**SA4**), both of which share similar  $IC_{50}$  values with CID-5932584 (**SA3**) *in vitro*.

These results suggest a beneficial role for the fluorine substituent (which is only present in CID-5932584 (**SA3**), maybe by facilitating better membrane permeability. The latter could be a limiting factor for these compounds, considering the substantially higher concentration that is needed to see clear effects in cells vs. inhibition of recombinant protein. Nonetheless, the fact that these inhibitors are not toxic to cells with low levels of endogenous VHR, such as primary normal keratinocytes, these compounds may well be a starting point for development of drugs for the treatment of cervical cancer and perhaps other cancers. Indeed, our results provide first evidence that pharmacological inhibition of VHR could be beneficial in treating such diseases. However, additional studies will be necessary to get better insights into the role of VHR phosphatase in cell cycle regulation and cancer and to test the activity of these compounds *in vivo* using mouse models.



**Figure 4.** Effects of VHR inhibitors on proliferation and survival of cervix cancer cells. (A) Cell numbers of HeLa cervix carcinoma cells and normal keratinocytes (NK) after 24 h of treatment with 20  $\mu$ M inhibitors, given as percentage of the DMSO control. (B,C) [<sup>3</sup>H]-Thymidine incorporation assay with 20  $\mu$ M inhibitors or vehicle (DMSO) in HeLa (B) or Caski (C) cervix cancer carcinoma cells. (D) Survival/death of HeLa and Caski cells in presence of 20  $\mu$ M inhibitors or DMSO after 24 h, evaluated by Annexin V FITC/ PI flow cytometry assay (FACS Vantage, BD). Percentage of live cells (Annexin V negative and PI negative cells on flow cytometry dot plots) is given. All data are reported as mean  $\pm$  SD from three independent experiments. (see ref. 18)

#### h. Detailed synthetic pathway for making probe

Compound is obtained commercially, but BCCG did prepare a synthetic route.

#### i. Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.)

This probe has demonstrated low micromolar potency for VHR and selectivity against HePTP and MKP-1. In PubChem this compounds has been tested in only the SAR assays associated with the VHR project.

While the probe compound CID6161281 [**ML113**] (MLS-0425632) contains a sulfonic acid that is expected to be fully negatively charged, however, it is also a very hydrophobic molecule. Consistent with these characteristics, it exhibited low solubility and permeability at all pHs tested (**Table 8**). It exhibits high plasma protein binding (both human and mouse). However, it has good stability in both human and mouse plasma. But, it has some instability in the presence of both human and mouse microsomes (determined by the exploratory pharmacology group). The probe compound has a LD<sub>50</sub> >50uM towards Fa2N-4 immortalized human hepatocytes.

<b>Table 8.</b> Summary of <i>in vitro</i> ADMET/PK Properties of HePTP Inhibitor Probe							
Probe CID Probe ML# BCCG MLS-#	Aqueous Solubility (µg/mL) <sup>a</sup> (@ pH)	PAMPA Pe (x10 <sup>-6</sup> cm/s) <sup>b</sup> (@ pH)	Plasma Protein Binding (% Bound)		Plasma Stability <sup>c</sup> Human Mouse/ Mouse/	Hepatic Microsome Stability <sup>d</sup> Human/ Mouse	Hepatic Toxicity <sup>e</sup> LC50 (µM)
			Human 1µM/ 10µM	Mouse 1µM/ 10µM			
CID6161281 <b>ML113</b> MLS-0425632	0.16 (5.0) 0.46 (6.2) 0.65 (7.4)	<0.8 (5.0) <0.8 (6.2) <0.9 (7.4)	98.28/ 99.83	84.59/ 70.16	87.89/ 100	57.85/ 51.84	>50

<sup>a</sup> in aqueous buffer, pH's 5.0/6.2/7.4

<sup>b</sup> in aqueous buffer; Donor compartment pH's 5.0/6.2/7.4; Acceptor compartment pH 7.4

<sup>c</sup> % remaining at 3 hr

<sup>d</sup> % remaining at 1 hr

<sup>e</sup> towards Fa2N-4 immortalized human hepatocytes

#### j. A tabular presentation summarizing known probe properties

<b>Table 9.</b> Properties computed from Structure	
CID6161281 [ <b>ML113</b> ] (MLS-0425632)	
Molecular Weight [g/mol]	612.13938
Molecular formula	C <sub>28</sub> H <sub>22</sub> ClN <sub>3</sub> O <sub>5</sub> S <sub>3</sub>
XLogP3-AA	5.6
H-Bond Donor	1
H-Bond Acceptor	6
Rotatable Bond Count	9
Exact Mass	611.041011
MonoIsotopic Mass	611.041011
Topological Polar Surface Area	168
Heavy Atom Count	40
Formal Charge	0
Complexity	1030
Isotope Atom Count	0
Defined Atom StereoCenter Count	0
Undefined Atom StereoCenter Count	0
Defined Bond StereoCenter Count	1
Undefined Bond StereoCenter Count	0

Covalently-Bonded Unit Count	1
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**4. Appendices (useful in a tabular form):**

**a. Comparative data on (1) probe, (2) similar compound structures (establishing SAR) and (3) prior probes**

Already summarized above in **Tables 1, 2, 5 and 6**

**b. Comparative data showing probe specificity for target**

Already summarized above in **Table 6**

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