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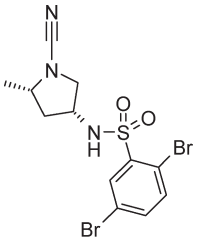
Discovery of Novel Cyanamide-Based Inhibitors of Cathepsin C

Dramane Lainé,* Michael Palovich, Brent McCleland, Emilie Petitjean, Isabelle Delhom, Haibo Xie, Jianghe Deng, Guoliang Lin, Roderick Davis, Anais Jolit, Neysa Nevins, Baoguang Zhao, Jim Villa, Jessica Schneck, Patrick McDevitt, Robert Midgett, Casey Kmett, Sandra Umbrecht, Brian Peck, Alicia Bacon Davis, and David Bettoun

GlaxoSmithKline, Respiratory CEDD, 709 Swedeland Road, P.O. Box 1539, King of Prussia, Pennsylvania 19406-0939, United States

ABSTRACT The discovery of potent and selective cyanamide-based inhibitors of the cysteine protease cathepsin C is detailed. Optimization of the template with regard to plasma stability led to the identification of compound **17**, a potent cathepsin C inhibitor with excellent selectivity over other cathepsins and potent in vivo activity in a cigarette smoke mouse model.

KEYWORDS Cathepsin C inhibitors, cyanamides, potent, selective, cigarette smoke



17

Cathepsin C (also known as dipeptidyl peptidase I, hDPPI, EC: 3.4.14.1) is a lysosomal cysteine dipeptidyl aminopeptidase from the papain family of proteases. As its name suggests, cathepsin C removes dipeptide moieties from the N termini of target proteins.[1,2] The enzyme is constitutively produced in many tissues with the highest expression levels found in lung, kidney, liver, and spleen.[3] There is increasing evidence that cathepsin C plays a key role in a variety of diseases, including sepsis,[4] arthritis,[5] and other inflammatory disorders.[6−12] From knockout mice studies, it appears that cathepsin C is coexpressed and acts as a physiological activator of the neutrophil-derived serine proteases: neutrophil elastase, cathepsin G and proteinase 3,[5] the mast cell chymase and trypase,[13] and the lymphocytes derived granzymes A and B.[14] Once activated, these proteases are capable of degrading various extracellular matrix components, which can lead to tissue damage and chronic inflammation. Thus, inhibitors of cathepsin C could potentially be useful therapeutics for the treatment of neutrophil-dominated inflammatory diseases such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF).[15,16]

An X-ray crystal structure of cathepsin C has been determined and showed that, unlike other cysteine proteases, the enzyme is composed of four identical subunits, each containing three chains: a heavy chain, a light chain, and an exclusion domain.[17] A catalytic ion pair near the scissile peptide bond is formed by the Cys234 and His381 residues.[18] The S1 site is not a well-defined pocket but rather a surface located near the solvent surface. The S2 pocket is deep and hydrophobic and contains a chloride ion and two solvent molecules at the bottom. The N-terminal Asp 1 residue of the exclusion domain is placed at the entrance of the S2 pocket. The exclusion domain is unique to cathepsin C and key to the substrate specificity of the enzyme as it prevents endopeptidase activity by blocking substrate access beyond the S2 site. The requirements for substrate recognition and cleavage by cathepsin C have been established and showed that the enzyme can accept a broad variety of peptide substrates.[1,2,19,20] The crystal structure of cathepsin C with a covalent inhibitor has also been reported.[21] Because of the positioning of the S1 site on the surface of the enzyme, P1 residues bearing a hydrophobic, basic, acidic, or polar side chain are accommodated. Aliphatic, hydrophobic, polar, and acidic residues are accepted in the S2 pocket with a preference for small aliphatic residues. The presence of a terminal N-amino group is necessary to anchor the substrate in the active site via an ionic interaction with Asp1. The enzyme cleaves two-residue units from the substrate until it reaches a stop sequence, which could be an arginine or lysine in P2, a proline in P1 or P1′, or an isoleucine in P1.

A variety of peptidyl derivatives have been reported in the literature as cathepsin C inhibitors. It comprised mainly di- or tripeptide derivatives functionalized with an electrophilic group, such as a vinyl sulfone,[22−24] an acyloxymethyl ketone,[23] a fluoromethyl ketone,[23] a conjugated ester,[24] a nitrile,[24−26] a phosphine group,[27] a semicarbazide moiety,[28] or a diazomethylketone.[29] A few peptides,[30−32] or the nonspecific cysteine protease inhibitor, E64, have also been found to inhibit cathepsin C.[32] To date, nonpeptidic inhibitors of cathepsin C have not been disclosed. Several series of cyanamide-based inhibitors of cysteinyl cathepsins have been previously reported from these laboratories[33] or elsewhere.[34,35] These compounds displayed nanomolar activity for cathepsins K or L, but no activity for cathepsin C has ever been disclosed. On the basis

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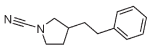
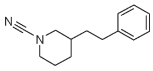
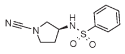
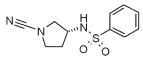
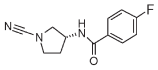
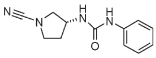
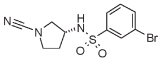
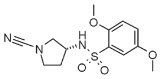
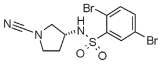
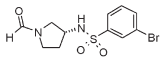
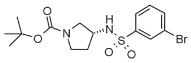
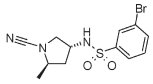
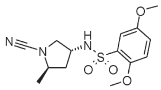
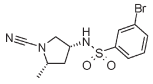
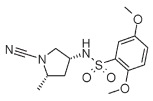
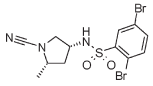
Table 1. Illustrative Examples of SAR Study

Cpd #	Structure	Cat C pIC_{50}	Cat B pIC_{50}	Cat K pIC_{50}	Cat L pIC_{50}	Cat S pIC_{50}	Rat Plas. St. (% left @30 mins)
1		5.7	5.3	5.9	6.2	5.8	13
2		< 4.6	<4.6	5.4	5.8	4.6	NT [a]
3		< 4.6	NT	5.0	NT	5.1	NT
4		6.5	5.4	7.5	NT	6.3	48
5		6.5	5.0	6.7	NT	5.8	30
6		5.6	5.4	7.2	NT	4.9	NT
7		6.0	5.9	NT	5.9	5.4	NT
8		8.1	5.2	8.1	7.4	6.5	18
9		8.0	4.9	7.1	5.9	5.7	23
10		> 9.4	5.8	8.7	7.6	6.9	18
11		< 4.6	<4.6	5.3	4.9	<4.6	95[b]
12		< 4.6	< 4.6	< 4.6	< 4.6	< 4.6	≥ 100
13		4.8	<4.6	6.0	5.6	4.8	45[b]
14		<4.6	<4.6	<4.6	<4.6	<4.6	88
15		6.8	<4.6	6.6	5.5	5.4	NT
16		6.9	<4.6	6.2	5.5	4.8	NT
17		8.7	<4.6	7.4	6.3	6.0	88[b]

[a] NT, not tested. [b] Values are the mean of two or more independent assays.

of that knowledge, we decided to examine the potential of cyclic cyanamides as cathepsin C inhibitors. Our aim was to identify molecules that could be further evaluated in vivo in a cigarette smoke mouse model. These compounds needed to be selective over the structurally related cysteine proteases: cathepsins L, S, B, and K, which have all been reported to play vital roles in number of important physiological and pathological processes.[36]

An initial set of compounds was rapidly prepared by cyanation of cyclic amine building blocks, available either

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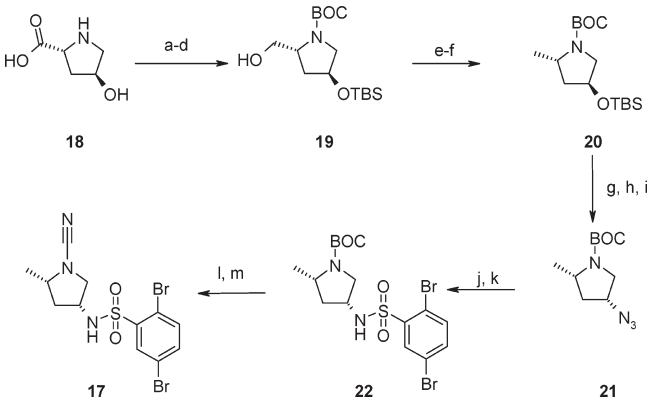
from commercial sources or from our compound collection. Illustrative compounds of this initial structure−activity relationship (SAR) study are shown in Table 1. The 3-phenethyl derivative **1** displayed an encouraging basal cathepsin C activity with a pIC_{50} of 5.7 but no selectivity over the other cathepsins, whereas the unsubstituted pyrrolidine nitrile **2** and the piperidine analogue **3** were inactive. These results prompted us to prepare a variety of 3-substituted pyrrolidine nitriles to examine different linkers between the 5-membered ring and the phenyl group. This approach led to the identification of the sulfonamine enantiomers **4** and **5**. Both molecules displayed similar activity at cathepsin C (pIC_{50} 6.5), which was slightly superior to that of the corresponding amide **6** or urea **7**. Because of the higher selectivity of **5** over other cathepsins, the S configuration at C-3 appeared more promising and was thus selected for further lead optimization. Further functionalization of the sulfonamide phenyl led to the identification of more potent molecules, such as the bromo derivative **8** and the dimethoxy **9** (pIC_{50} of 8.1 and 8.0, respectively). Subsequent replacement of the methoxy group of **9** with bromide atoms gave **10** with excellent cathepsin C activity (pIC_{50} > 9.4) and good selectivity. Metabolic stability, however, turned out to be a problem for **8**, **9**, and **10** as the compounds displayed high rat plasma turnover (< 25% of compound remaining after 30 min of incubation, Table 1) despite being stable in human plasma (data in the Supporting Information). As our primary in vivo models were based in rodent, we needed to ascertain the reason for this instability. Our initial suspicions were that part of the instability was due to the high reactivity of the nitrile warhead. To test this hypothesis, compounds bearing an aldehyde (**11**) and a BOC group (**12**) in place of the nitrile moiety were examined. We found that although neither compound inhibited cathepisn C, both were indeed stable in rat plasma. Studies were then initiated to ascertain the mechanism of the instability. To identify the cause of the degradation of the compounds in the rat, a series of experiments examining the stability of **8**, in the presence of various additives, were conducted. No degradation was observed in the presence of rat serum albumin, free cysteine, or glutathione or when **8** was incubated in the presence of a broad spectrum esterase inhibitor (see the graphs in the Supporting Information). We concluded from these studies that the instability of the compounds in rat plasma probably involved an esterase acting on the nitrile group. Next, we hypothesized that we could reduce the rate of reaction with the esterase by reducing the electrophilicity and steric accessibility of the nitrile head. To this end, we examined the introduction of a methyl group at the C-5 position while keeping the optimal C-3 configuration. As shown in Table 1, this approach led to the identification of the (5*R*)-methyl derivatives **13** and **14** with improved plasma stability. Both compounds, however, did not inhibit cathepsin C and had little or no activity at other cathepsins. The corresponding (5*S*)-methyl derivatives **15** and **16** were also prepared and displayed much better activity at cathepsin with pIC_{50} values of 6.8 and 6.9, respectively. In an analogous manner to the previously described functionalization of **5**, the introduction of 2,5-dibromo substituents on the phenyl sulfonamide group led to a significant

Scheme 1[a]



[a] Reagents and conditions: (a) MeOH, AcCl. (b) $(BOC)_2O$, TEA. (c) TBSCl, imidazole. (d) $LiBH_4$. (e) MsCl, TEA. (f) $LiEt_3BH$. (g) TBAF, THF. (h) Same as e. (i) $tBuN^+N_3^-$, CH_3CN. (j) H_2, Pd/C, MeOH. (k) 2,5-DiBrPhSO$_2$Cl, TEA. (l) 4 N HCl, dioxane. (m) BrCN, DCM.

increase in potency, and **17** was identified with a pIC_{50} of 8.5 and good stability in rat plasma. At that point of our investigation, the decision was made to focus on a mouse in vivo model for further pharmacological evaluation. The stability of **17** was therefore assessed in mouse plasma and found to be somewhat lower (57% of compound remaining after 30 min) than in rat. Although suboptimum, this level of stability was deemed to be adequate for further in vivo pharmacological evaluation.

The methods of preparation of compounds **1**−**16** are shown in the Supporting Information. Compound **17** was synthesized according to the route outlined in Scheme 1. Esterification of (4*S*)-4-hydroxy-D-proline **18** in acidic methanol, followed by successive conversion of the amine to the corresponding BOC derivative then of the secondary alcohol to a TBS ether gave, after reduction of the methyl ester moiety, the primary alcohol **19**. Subsequent conversion of **19** to the corresponding methanesulfonate ether followed by treatment with lithium triethyl borohydride produced the (5*S*)-methyl derivative **20**. Further desilylation of **20** with TBAF gave a secondary alcohol intermediate, which was transformed to the azide **21** with inversion of configuration at C-3 by activation of the alcohol as a methanesulfonate ether and treatment with *t*-butyl ammonium azide. Palladium-catalyzed hydrogenation of **21** followed by treatment with 2,5-dibromobenzenesulfonyl chloride gave the derivative **22**. Subsequent removal of the BOC protecting group with 4 N HCl in dioxane, followed by treatment with cyanogen bromide, resulted in the formation of **17**.

The cocrystal structure of **17** bound in the cathepsin C active site indicates that the nitrile covalently reacts with catalytic Cys234 forming a thioimidate complex with a hydrogen bond to oxyanion hole residue Gln228 (Figure 1). The sulfonamide forms a hydrogen bond to the backbone NH of Gly277 and links the pyrrolidine nitrile template to the 2,5-dibromophenyl moiety, which trajects into the S2 pocket. The 5-methyl restricts the conformation of the pyrrolidine ring and interacts with the back wall of the binding site.

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The in vivo pharmacokinetic (PK) properties of **17** were assessed in several species as shown in Table 2. In the mouse, **17** displayed variable PKs. In three out of four mice dosed orally, low exposure and low bioavailability were observed, whereas one showed high bioavailability and ~9-fold higher exposure. Variable data also were obtained with regard to plasma clearance when eight separate mice were administered **17** intravenously with a 50% frequency of mice demonstrating high and low plasma clearance of **17**, respectively. Similar results were observed with other structurally related pyrollidine nitriles (data not shown) and may be indicative of polymorphic metabolism-mediated clearance of this class of compounds. In an attempt to determine whether these findings were mouse-specific, we also examine **17** in the rat and dog. In rat, the compound demonstrated low oral exposure and bioavailability, likely due to high plasma clearance. Overall, the PK profile of **17** was improved in dog as compared to rat and to the apparent extensively metabolized mouse population with moderate clearance, systemic exposure, and bioavailability observed consistently across all of the dogs evaluated.

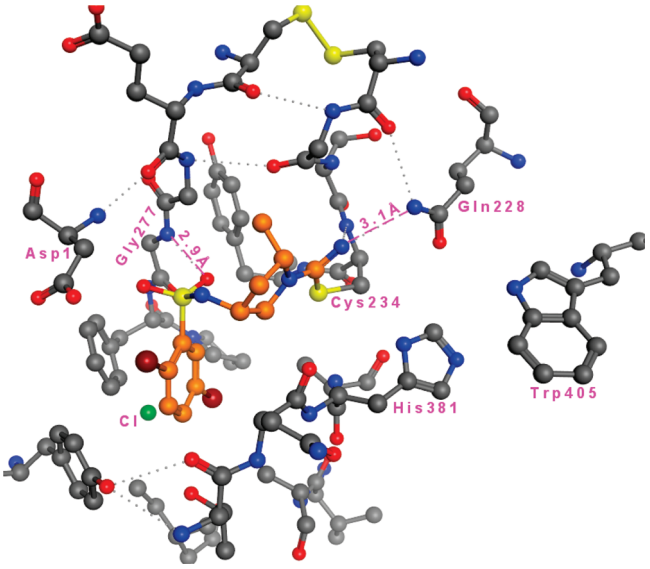

Figure 1. X-ray crystal structure of **17**.

The effect of oral administration of compound **17** on cathepsin C inhibition was examined in mice (Figure 2). Time−course activity studies at a high dose (30 mg/kg) showed statistically significant inhibition (35%) of cathepsin C activity by **17** in plasma at 30 min (Figure 2a) and in lung homogenate at 0.5, 1.5, and 3 h (32, 16, and 28%, Figure 2b). The compound was not detected in plasma at any of the time points of the plasma study. These results showed that in vivo inhibition of cathepsin C by **17** is achievable after oral dosing; yet, the level of engagement of the enzyme might be suboptimal because of the variable PK profile and short half-life (20 min) of **17** in mice. To bypass microsomal clearance and potentially observe greater effects in the lung, it was decided to dose topically to the lung via intranasal delivery. When dosed intranasally at 30 mg/kg, **17** showed a significant time-dependent inhibition of cathepsin C activity in lung homogenate (53, 66, and 85% at 0.5, 1.5, and 3 h), which was much greater than when a similar dose was administered orally (Figure 3a). At the optimum time point of 30 min, compound **17** demonstrated a dose-related inhibition of cathepsin C with an ED50 of 20 mg/kg (Figure 3b). These results demonstrated that intranasal delivery was a superior route of administration for **17** with respect to the

Table 2. PK Profile of **17**

species	dose (iv/po; mg/kg)	F^b (%)	AUC (po, ng h/mL)	Vdss (L/kg)	Clp[c] (mL/min/kg)	$t_{1/2}$ (h)
rat[a]	1/2	8 ± 3	7 ± 4	3.1 ± 2.0	162 ± 14	0.2 ± 0.1
mouse[d]	5/20	1, 7, 75, 2	9, 11.5, 89, 10	0.4, 1.8, 1.8, 0.9	16, 90, 137, 33	0.4, 0.2, 0.2, 0.3
dog[a]	5/10	20 ± 7	1180 ± 606	1.3 ± 0.2	29 ± 9	0.7 ± 0.1

[a] Values are the mean of two or more independent assays. [b] F, percent bioavailability. [c] Clp, systemic plasma clearance. [d] Individual values listed due to variability.

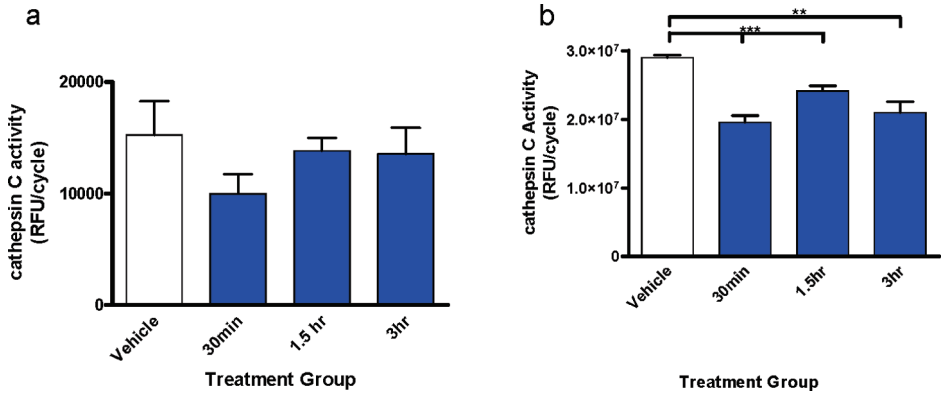

Figure 2. In vivo effect of **17** following oral administration. (a) Time course of cathepsin C activity in plasma as measured by the cleavage of $(H\text{-}Gly\text{-}Arg)_2$ R110 (10 μM) in mice administered orally with **17** (30 mg/kg, $n = 5$). (b) Time course of cathepsin C activity as measured by the cleavage of $(H\text{-}Gly\text{-}Arg)_2$ R110 (10 μM) in crude lung homogenate from C57BL/6J mice administered orally with **17** (30 mg/kg, $n = 5$).

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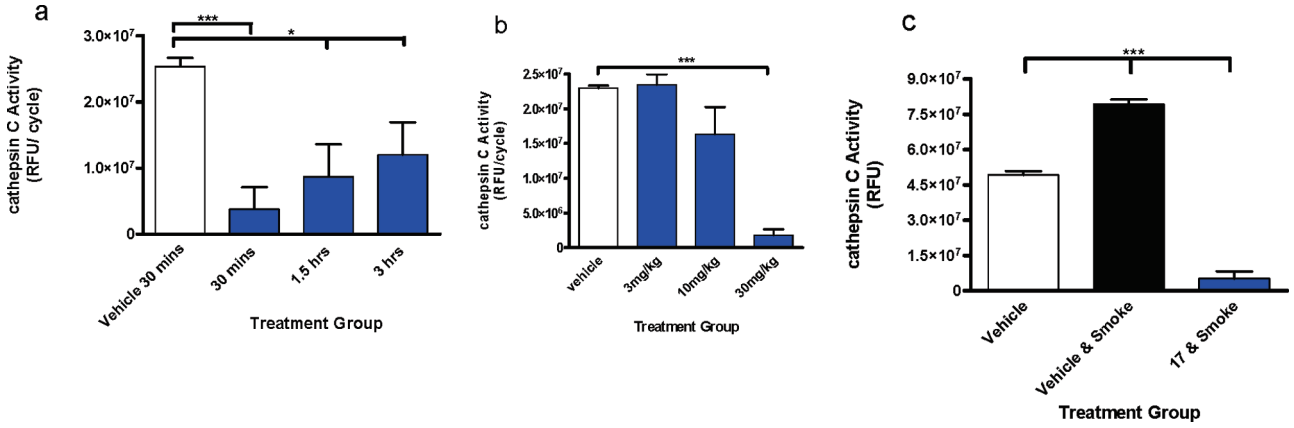

Figure 3. In vivo effect of **17** following intranasal administration. (a) Time course of cathepsin C activity as measured by the cleavage of $(H\text{-}Gly\text{-}Arg)_2$ R110 in crude lung homogenate from C57BL/6 mouse lungs collected after intranasal treatment with **17** ($n = 5$). (b) Thirty minute dose response of **17** administered to C57BL6/J mice intranasally and measured in crude lung homogenate ($n = 5$). (c) Cathepsin C activity as measured by the cleavage of $(H\text{-}Gly\text{-}Arg)_2$ R110 (10 μM) in crude lung homogenate from BALB/c mice after exposure to cigarette smoke for 3 days and **17** (30 mg/kg) administered intranasally daily for 4 days ($n = 5$).

level of engagement of the enzyme in the lung, and the decision was made to use this mode of delivery to evaluate the compound in a 5 day cigarette smoke mouse model (Figure 3c). In this study, mice were administered **17** on day 1 (30 mg/kg) and then for 3 further days were daily administered similar doses of **17** immediately followed by cigarette smoke inhalation. On the fifth day, lung homogenate was collected and evaluated. A significant increase in cathepsin C activity induced by cigarette smoke was observed in the vehicle and smoke-treated animal group. The level of cathepsin C activity of the **17**-treated group was significantly reduced to levels below that of the vehicle-only treated group. Taken together, these data indicate that compound **17** is an effective cathepsin C inhibitor in mice when administered intranasally.

In conclusion, a novel series of cyanamide-based inhibitors of the cysteine protease cathepsin C were discovered. After optimization of the structural features with regard to plasma stability, **17** was identified as a potent cathepsin C inhibitor with excellent selectivity over other cathepsins. Crystallographic studies with **17** showed that a covalent bond was formed between the nitrile group and the Cys 234 residue in the active site of the enzyme. Because of its variable PK profile and short half-life, **17** displays minimal activity in mice when administered orally. In a cigarette smoke model, the increased activity of cathepsin C was significantly reduced by intranasal administration of **17**. The significance of these findings is currently being investigated in our laboratory. Further accounts on this novel series will be the subject of future publications.

SUPPORTING INFORMATION AVAILABLE General methods of preparation of compound **1**−**22** (Table 1 and Scheme 1), general methods for the recombinant cathepsins in vitro assays (Table1), procedures for the plasma stability studies (Table 1), data for the human plasma stability studies and the graphs of the rat plasma stability studies for compounds **8**, **11**, and **12**, protocols for the in vivo experiments described in Figures 2 and 3, and exposure levels for Figure 2a. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes: The atomic coordinates for the X-ray crystal structure of **17** have been deposited with the RCSB Protein Data Bank under the accession code 3PDF.

AUTHOR INFORMATION

Corresponding Author: *Tel: 1-610-270-7889. Fax: 1-610-270-4451. E-mail: dramane.i.laine@gsk.com.

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