Appendix B: Apredica ADMET Report

(Supplemental Information Continued from Chapter 3)

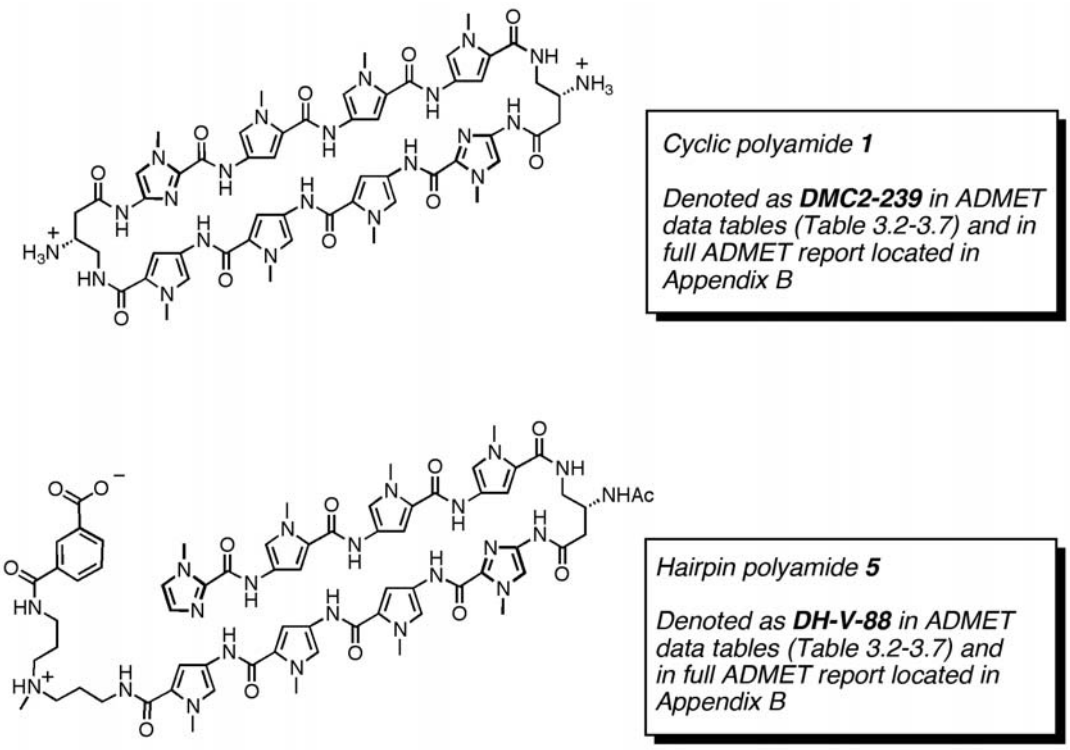

Figure B.1 Polyamides **1** and **5** were subjected to preclinical ADMET testing by contract service at Apredica (Watertown, MA). Shown in Chapter 3 of this thesis (Table 3.2-3.7) are summaries of the ADMET results taken directly from the final report provided by Apredica. The full ADMET report, which includes experimental conditions, is contained on the following pages.

Apredica Study Number: CIT-001

ADMET Properties of Test Agents

Final Report

Sponsor:	California Institute of Technology Division of Chemistry and Chemical Engineering 1200 E California Blvd; MC 164-30 Pasadena, CA 91125 (USA)
Sponsor's Representative	Daniel Harki, Ph.D. Phone: (626) 395-6032 Email: harki@caltech.edu
Test Facilities:	Apredica 313 Pleasant Street Watertown, MA 02472 Telephone Number: 617.812.1911
Study Director	Jon Gilbert In Vitro ADMET, Bioanalysis 617.812.1911x122 jon@apredica.com

Approved: Jon Gilbert

Date: 12/2/08

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1 Objective

The objective of this study is to determine the ADMET properties of test agents.

1.1 Regulatory Guidelines

This study was not conducted under US FDA Good Laboratory Practice Regulations (GLPs). Standard operating procedures of Apredica were used throughout the study.

2 Test Articles

Apredica ID	Client ID	Physical Form	Submitted FW	Parent MW	Stock solutions
CIT-001-01	DH-V-88	Solid	1399	1399	50 mM DMSO
CIT-001-02	DMC2-239	Solid	1407.26	1178.5	50 mM DMSO

Test agent powders were stored at -20 °C. Stock solutions were stored at -20 °C.

3 Test Methods

Testing was performed at Apredica in Watertown, MA.

3.1 Analytical Methods

3.1.1 Method development

The signal was optimized for each compound by ESI positive or negative ionization mode. A MS2 scan was used to identify the precursor ion and a product ion analysis was used to identify the best fragment for analysis and to optimize the collision energy. An ionization ranking was assigned indicating the compound's ease of ionization.

3.1.2 Analysis

Samples were analyzed by LC/MS/MS using either an Agilent 6410 mass spectrometer coupled with an Agilent 1200 HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software (Agilent), or an ABI2000 mass spectrometer coupled with an Agilent 1100 HPLC and a CTC PAL chilled autosampler, all controlled by Analyst software (ABI). After separation on a C18 reverse phase HPLC column (Agilent, Waters, or equivalent) using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS) using ESI ionization in MRM mode.

3.2 In vitro *ADME-Tox Experimental Conditions*

Additional protocol details are given in Appendix A.

3.2.1 Caco-2 monolayer permeability experimental conditions

Apredica ID	Client ID	Test conc.	Assay Time	Direction	Reference compounds	Analytical method
CIT-001-01	**DH-V-88**			A->B	warfarin	
CIT-001-02	**DMC2-239**	10 μM	2 hr	B->A	ranitidine	LC/MS/MS

3.2.2 Cytotoxicity experimental conditions

Apredica ID	Client ID	Test conc.	Assay time	Cell lines	Readout	Reference compound	Analytical method
CIT-001-01 **CIT-001-02**	**DH-V-88** **DMC2-239**	100, 40, 16, 6.4, 2.6, 1.0, 0.4, 0.16, 0.07 μM	48 hr	HepG2 NIH/3T3	Neutral red	chlorpromazine propranolol	fluorescent plate reader

3.2.3 Fluorescent cytochrome P450 inhibition experimental conditions

Apredica ID	Client ID	Test conc.	Cyp assays	Reference compound	Analytical method
		50, 16.7, 5.6, 1.9, 0.6, 0.2, 0.07, 0.02 μM	Cyp1A2/CEC Cyp2C8/DBP Cyp2C9/DBF Cyp2C19/DBF Cyp2D6/AMMC Cyp3A4/DBF Cyp3A4/BFC	α-naphthoflavone ketoconazole sulphaphenazole tranylcypromine quinidine ketoconazole ketoconazole	fluorescent plate reader
CIT-001-01	**DH-V-88**				
CIT-001-02	**DMC2-239**				

3.2.4 Microsomal stability experimental conditions

Apredica ID	Client ID	Test conc.	Micro-some source	Protein conc.	Incub-ation	Ref. comp.	Analytical method
					0, 10, 20, 40, and 60 min 37 °C	verapamil warfarin	LC/MS/MS
CIT-001-01	**DH-V-88**		Human and rat	0.3 mg/mL			
CIT-001-02	**DMC2-239**	5 μM					

3.2.5 Plasma stability experimental conditions

Apredica ID	Client ID	Test conc.	Plasma source	Incub-ation	Reference compounds	Analytical method
				0, 15, 30, 60, 120 min 37 °C	propantheline	LC/MS/MS
CIT-001-01	**DH-V-88**		Human and rat			
CIT-001-02	**DMC2-239**	10 μM				

3.2.6 Plasma protein binding experimental conditions

Apredica ID	Client ID	Test conc	Plasma species	Incub-ation	Sep. method	Ref. compound	Analytical method
CIT-001-01	**DH-V-88**						
CIT-001-02	**DMC2-239**	10 μM	Human and rat	4 hr 37 °C	equilibrium dialysis	warfarin, atenolol	LC/MS/MS

3.2.7 hERG FastPatch experimental conditions

Apredica ID	Client ID	Test conc	Medium	Incub-ation	Ref. comp.	Analytical method
		100, 30, 10, 3, 1, 0.3, 0.1, 0.03 μM	HEPES-aspartate buffer	5 min ambient temp.	E-4031	electro-physiology
CIT-001-01	**DH-V-88**					
CIT-001-02	**DMC2-239**					

4 Results

4.1 Analytical

4.1.1 Method development

Client ID	MW	Polari-zation	Precursor m/z	Product m/z	Collision energy (V)	Ionization classification[a]
DH-V-88	1399	pos	700.2	231	26	2
DMC2-239	1178.5	pos	590.1	372	20	2

[a]Ionization classification:
1 = Highly ionizable
2 = Intermediately ionizable
3 = Poorly ionizable

The full scan mass spectrum, the product ion spectrum, and a sample chromatogram are shown in Appendix B.

4.2 In vitro *ADME-Tox Summary*

4.2.1 Caco-2 permeability summary

Client ID	test conc (μM)	Assay duration (hr)	mean A->B P_{app} [a] (10^{-6} cm s^{-1})	mean A->B P_{app} [a] (10^{-6} cm s^{-1})	Asymmetry ratio[b]	comment
Warfarin	50	2	35.4	7.9	0.2	high permeability control
Ranitidine	50	2	1.4	2.4	1.7	low permeability control
DH-V-88	10	2	ND	0.11	UD	
DMC2-239	10	2	ND	ND	ND	

[a]Apparent permeability
[b]P_{app}(B->A) / P_{app}(A->B)
ND = no compound detected in receiver solution

4.2.2 Cytotoxicity summary

Client ID	Cell line	IC50 (μM)	comment
Chlorpromazine	HepG2	13	Higly cytotoxic control
Propranolol	HepG2	80	Low cytotoxic control
DH-V-88	HepG2	>100	
DMC2-239	HepG2	>100	

4.2.3 Fluorescent Cyp IC50 summary

	IC_{50} (μM)						
Client ID	Cyp1A2 / CEC	Cyp2C8/DBF	Cyp2C9 / DBF	Cyp2C19 / DBF	Cyp2D6 / AMMC	Cyp3A4 / BFC	Cyp3A4 / DBF
Controls	0.2 α-naphtho-flavone	2.3 ketoconazole	1.1 sulpha-phenazole	5.6 tranyl-cypromine	0.05 quinindine	1.26 ketoconazole	1.26 ketoconazole
DH-V-88	>50	>50	>50	>50	>50	47.6	>50
DMC2-239	>50	>50	>50	>50	>50	37.7	>50

4.2.4 hERG FastPatch summary

Client ID	IC_{50} (μM)	comment
E-4031	99% at 0.5 μM	positive control
DH-V-88	>100	*
DMC2-239	>100	*

*The solubility limit for this experiment, as determined by vehicle controls, was 17.3 x 10^3 LSU (horizontal black line). Based on the data obtained, there may be solubility issues for both test articles at 30 and 100 μM in our physiological saline solution (HB-PS, 0.3%DMSO). Precipitation of DH-V-88 at 100 μM was visible to the naked eye.

4.2.5 Microsomal intrinsic clearance summary

Client ID	test conc (μM)	test species	NADPH-dependent CL_{int}[a] (μl min^{-1} mg^{-1})	NADPH-dependent $T_{1/2}$[b] (min)	NADPH-free CL_{int}[a] (μl min^{-1} mg^{-1})	NADPH-free $T_{1/2}$[b] (min)	comment
Verapamil	1	Human	411.3	5.6	0.6	>180	metabolized control
Verapamil	1	Rat	2276	1	0.0	>180	metabolized control
Warfarin	1	Human	0.0	>180	0.0	>180	non-metabolized control
Warfarin	1	Rat	0.0	>180	0.0	>180	non-metabolized control
DH-V-88	5	Human	0.0	>180	0.0	>180	
DH-V-88	5	Rat	0.0	>180	0.0	>180	
DMC2-239	5	Human	0.0	>180	0.0	>180	
DMC2-239	5	Rat	0.0	>180	0.0	>180	

[a]Microsomal Intrinsic Clearance
[b]Half-life

4.2.6 Plasma half-life summary

Compound	test conc (uM)	medium	T1/2 (min)	Fraction remaining, max time (%)	comment
Propantheline	10.0	Human Plasma	35.5	5.8%	control
Propantheline	10.0	Rat Plasma	149.0	51.6%	control
DH-V-88	10.0	Human Plasma	>120	95.6%	
DH-V-88	10.0	Rat Plasma	>120	94.0%	
DMC2-239	10.0	Human Plasma	>120	124.5%	
DMC2-239	10.0	Rat Plasma	>120	120.3%	

[a]Half-life

4.2.7 Plasma protein binding summary

Client ID	test conc (μM)	Assay duration	Species	Mean free fraction (%)	comment
Warfarin	10	4 hr	Human	0.73%	high binding control
Warfarin	10	4 hr	Rat	5.47%	high binding control
Atenolol	10	4 hr	Human	76.2%	low binding control
Atenolol	10	4 hr	Rat	84.7%	low binding control
DH-V-88	10	4 hr	Human	0.0015%	
DH-V-88	10	4 hr	Rat	0.0016%	
DMC2-239	10	4 hr	Human	0.0000%	
DMC2-239	10	4 hr	Rat	0.0040%	

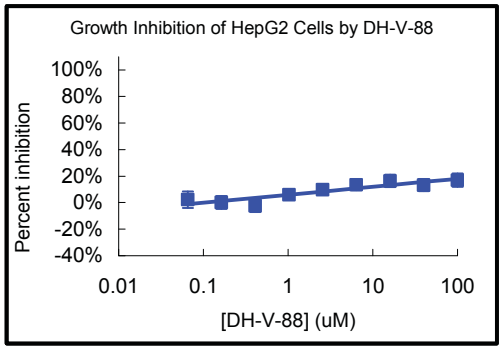
4.3 In vitro *ADME-Tox Individual Data*

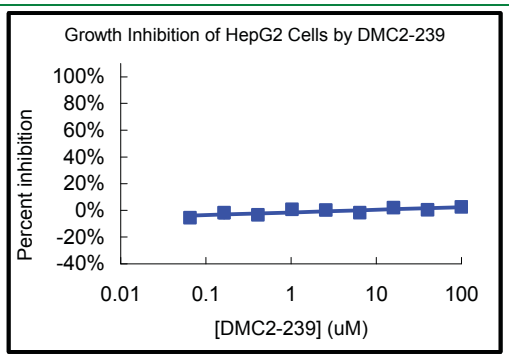
4.3.1 Caco-2 permeability individual data

Client ID	*test conc (μM)*	*direction*	*value*	*1st*	*2nd*	*mean*	*comment*
DH-V-88	10	A->B	dQ/dt[a]	ND	ND	ND	
		A->B	C_0[b]	1.1	1.0	1.1	
		B->A	dQ/dt[a]	8.6E-08	1.9E-08	5.3E-08	
		B->A	C_0[b]	1.4	1.4	1.4	
DMC2-239	10	A->B	dQ/dt[a]	ND	ND	ND	
		A->B	C_0[b]	0.05	0.02	3.5E-02	
		B->A	dQ/dt[a]	ND	ND	ND	
		B->A	C_0[b]	0.1	0.1	8.4E-02	

[a]rate of test agent permeation, area units/sec
[b]initial concentration (area units/cm^3)

4.3.2 Cytotoxicity individual data



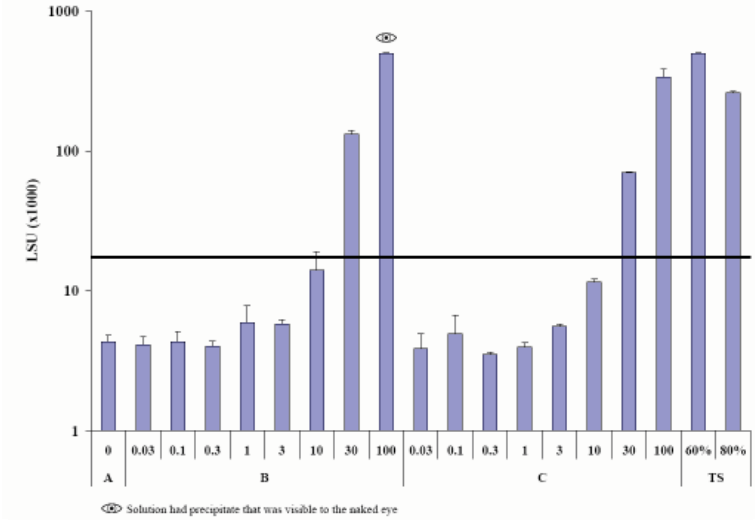


4.3.3 hERG FastPatch individual data

Test Article ID	IC50 (µM)	Conc. (µM)	Mean % hERG Inhibition	Standard Deviation	Standard Error	n	Individual Data Points (% Inhibition)
DH-88	> 100	0.03	4.6	3.8	2.7	2	7.2
							1.9
		0.1	5.6	3.1	2.2	2	7.8
							3.4
		0.3	1.9	4.0	2.9	2	-1.0
							4.7
		1	9.3	3.1	2.2	2	7.1
							11.5
		3	2.0	4.8	3.4	2	-1.4
							5.4
		10	3.1	2.3	1.6	2	1.6
							4.7
		30	1.2	0.7	0.5	2	1.6
							0.7
		100	0.9	1.4	1.0	2	-0.1
							1.9

Test Article ID	IC50 (µM)	Conc. (µM)	Mean % hERG Inhibition	Standard Deviation	Standard Error	n	Individual Data Points (% Inhibition)
DMG2	> 100	0.03	-1.2	0.1	0.1	2	-1.1
							-1.3
		0.1	2.1	0.4	0.3	2	2.3
							1.8
		0.3	-4.1	1.2	0.9	2	-3.2
							-4.9
		1	-3.7	3.3	2.4	2	-1.3
							-6.1
		3	-0.7	1.6	1.1	2	-1.9
							0.4
		10	-2.2	2.8	2.0	2	-4.2
							-0.2
		30	5.5	1.4	1.0	2	4.5
							6.5
		100	9.0	2.1	1.5	2	7.5
							10.5

TurboSol Figure 1

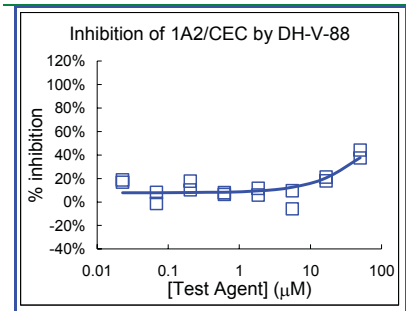


TurboSol Table 1

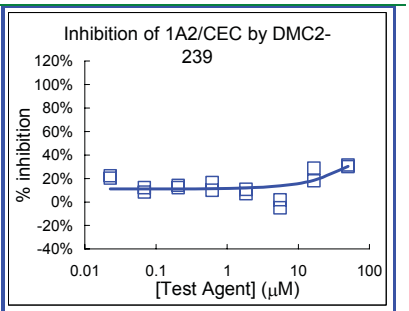
Compound	Figure Legend	Conc. (µM)	n	Average LSU (x1000)	Standard Deviation
Vehicle	A	0	11	6.1	6.2
88	B	0.03	3	4.1	0.6
		0.1	3	4.3	0.8
		0.3	3	4.0	0.4
		1	3	6.0	1.9
		3	3	5.8	0.5
		10	3	14.1	4.9
		30	3	131.9	7.4
		100	3	495.9	7.1
239	C	0.03	3	3.9	1.1
		0.1	3	5.0	1.7
		0.3	3	3.5	0.1
		1	3	3.9	0.3
		3	3	5.6	0.2
		10	3	11.6	0.5
		30	3	70.3	0.2
		100	3	335.9	46.3
Transmittance Standard	TS	60%	3	495.3	7.3
		80%	3	259.0	9.3

TS: Transmittance standard. % indicates percent light transmitted.
LSU: Light scatter unit

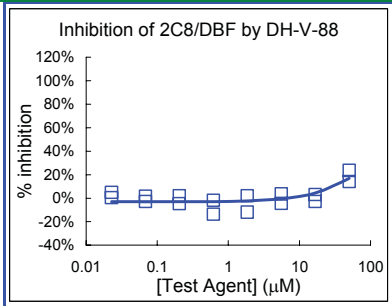
4.3.4 Fluorescent cytochrome P450 inhibition individual data



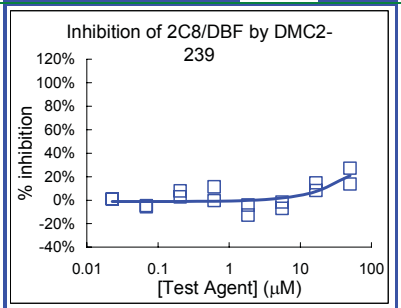
Test Agent:	DH-V-88		Assay:	1A2/CEC
IC50	>50		hill slope:	1.7
min	0%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	44%	37%	41%	38%
16.67	18%	21%	20%	21%
5.56	-6%	10%	2%	13%
1.852	6%	12%	9%	10%
0.617	8%	6%	7%	8%
0.2058	10%	18%	14%	8%
0.0686	8%	-1%	3%	8%
0.02286	17%	19%	18%	8%



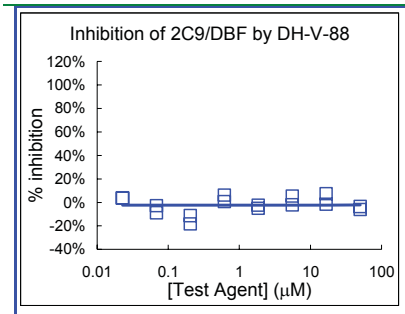
Test Agent:	DMC2-239		Assay:	1A2/CEC
IC50	>50		hill slope:	1.6
min	-6%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	32%	30%	31%	30%
16.67	29%	18%	23%	19%
5.56	2%	-5%	-2%	14%
1.852	11%	7%	9%	12%
0.617	16%	10%	13%	11%
0.2058	14%	12%	13%	11%
0.0686	12%	8%	10%	11%
0.02286	20%	22%	21%	11%



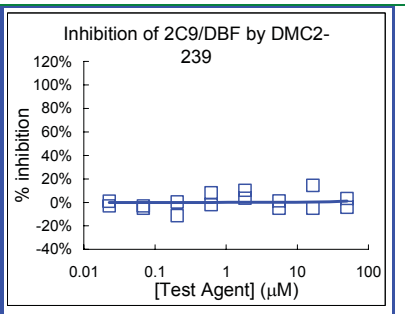
Test Agent:	DH-V-88		Assay:	2C8/DBF
IC50	>50		hill slope:	1.7
min	0%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	14%	24%	19%	17%
16.67	-3%	3%	0%	4%
5.56	-4%	4%	0%	0%
1.852	-12%	2%	-5%	-2%
0.617	-2%	-14%	-8%	-3%
0.2058	-5%	2%	-1%	-3%
0.0686	-3%	2%	-1%	-3%
0.02286	0%	5%	3%	-3%



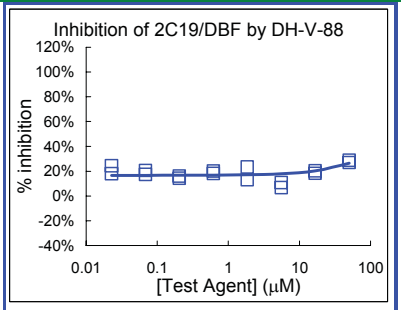
Test Agent:	DMC2-239		Assay:	2C8/DBF
IC50	>50		hill slope:	1.6
min	-6%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	27%	14%	20%	21%
16.67	8%	15%	11%	7%
5.56	-7%	-2%	-4%	2%
1.852	-4%	-13%	-9%	0%
0.617	11%	0%	6%	-1%
0.2058	8%	3%	5%	-1%
0.0686	-6%	-5%	-5%	-1%
0.02286	1%	1%	1%	-1%



Test Agent:	DH-V-88		Assay:	2C9/DBF
IC50	>50		hill slope:	1.7
min	0%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	-6%	-4%	-5%	-2%
16.67	7%	-2%	3%	-2%
5.56	-2%	5%	2%	-2%
1.852	-2%	-5%	-4%	-2%
0.617	1%	6%	3%	-2%
0.2058	-11%	-18%	-15%	-2%
0.0686	-9%	-3%	-6%	-2%
0.02286	4%	3%	4%	-2%



Test Agent:	DMC2-239		Assay:	2C9/DBF
IC50	>50		hill slope:	1.6
min	-6%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	3%	-4%	-1%	1%
16.67	-5%	14%	5%	0%
5.56	-5%	1%	-2%	0%
1.852	10%	3%	7%	0%
0.617	8%	-2%	3%	0%
0.2058	-11%	0%	-6%	0%
0.0686	-5%	-3%	-4%	0%
0.02286	-3%	1%	-1%	0%



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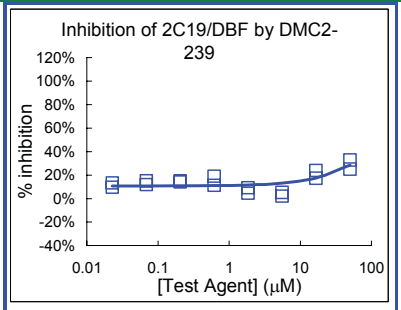
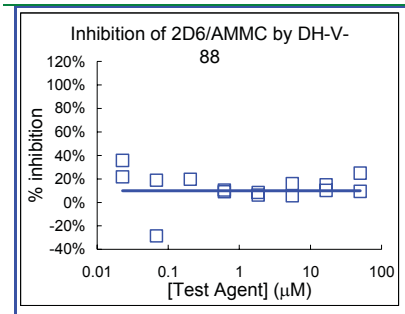
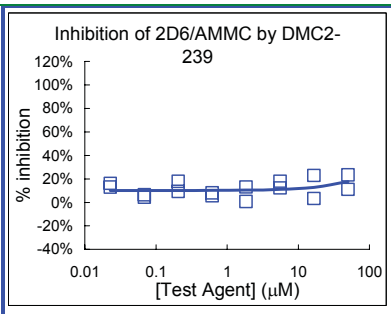
IC50	>50		hill slope:	1.7
min	0%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	27%	29%	28%	26%
16.67	20%	18%	19%	20%
5.56	7%	11%	9%	18%
1.852	23%	13%	18%	17%
0.617	20%	18%	19%	17%
0.2058	14%	16%	15%	17%
0.0686	17%	21%	19%	17%
0.02286	24%	18%	21%	17%



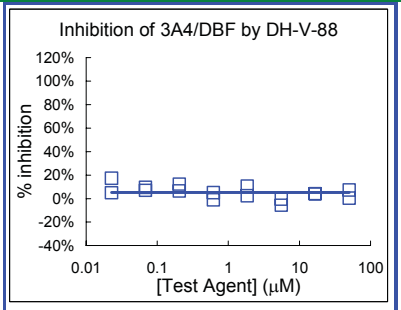
Test Agent:	DMC2-239		Assay:	2C19/DBF
IC50	>50		hill slope:	1.6
min	-6%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	33%	25%	29%	29%
16.67	17%	24%	21%	18%
5.56	5%	2%	4%	13%
1.852	5%	9%	7%	12%
0.617	11%	19%	15%	11%
0.2058	14%	15%	15%	11%
0.0686	12%	15%	13%	11%
0.02286	10%	13%	11%	11%



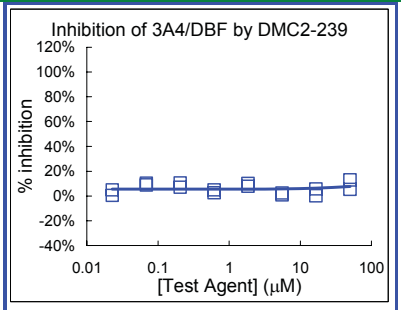
Test Agent:	DH-V-88		Assay:	2D6/AMMC
IC50	>50		hill slope:	1.7
min	0%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	9%	25%	17%	10%
16.67	15%	10%	13%	10%
5.56	6%	16%	11%	10%
1.852	8%	6%	7%	10%
0.617	10%	9%	10%	10%
0.2058		20%	20%	10%
0.0686	-29%	19%	-5%	10%
0.02286	36%	22%	29%	10%



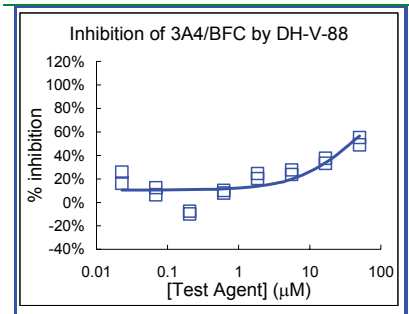
Test Agent:	DMC2-239		Assay:	2D6/AMMC
IC50	>50		hill slope:	1.6
min	-6%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	23%	11%	17%	18%
16.67	23%	3%	13%	13%
5.56	18%	12%	15%	11%
1.852	13%	1%	7%	10%
0.617	8%	6%	7%	10%
0.2058	18%	9%	14%	10%
0.0686	6%	4%	5%	10%
0.02286	16%	13%	15%	10%



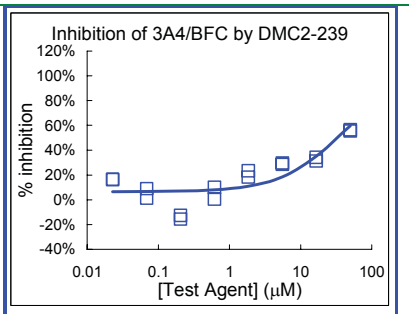
Test Agent:	DH-V-88		Assay:	3A4/DBF
IC50	>50		hill slope:	1.7
min	0%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	7%	0%	4%	5%
16.67	4%	4%	4%	5%
5.56	-6%	0%	-3%	5%
1.852	11%	2%	6%	5%
0.617	5%	-1%	2%	5%
0.2058	12%	6%	9%	5%
0.0686	9%	7%	8%	5%
0.02286	5%	17%	11%	5%



Test Agent:	DMC2-239		Assay:	3A4/DBF
IC50	>50		hill slope:	1.6
min	-6%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	13%	5%	9%	8%
16.67	6%	0%	3%	6%
5.56	2%	1%	2%	6%
1.852	8%	10%	9%	6%
0.617	5%	3%	4%	6%
0.2058	10%	7%	9%	6%
0.0686	9%	10%	10%	6%
0.02286	5%	0%	3%	6%

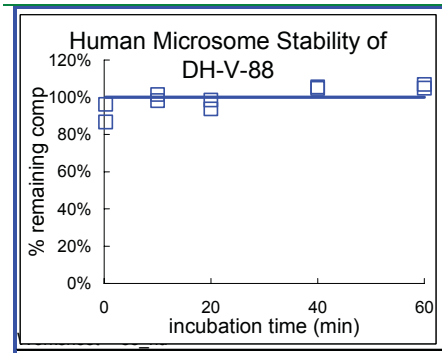


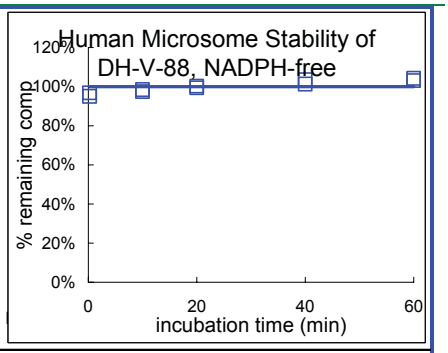
Test Agent:	DH-V-88		Assay:	3A4/BFC
IC50	48		hill slope:	1.7
min	0%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	49%	55%	52%	56%
16.67	33%	37%	35%	34%
5.56	28%	24%	26%	20%
1.852	20%	24%	22%	14%
0.617	8%	10%	9%	12%
0.2058	-8%	-10%	-9%	11%
0.0686	7%	12%	9%	11%
0.02286	16%	26%	21%	11%



Test Agent:	DMC2-239		Assay:	3A4/BFC
IC50	38		hill slope:	1.6
min	-6%		max	100%
[TA]	%Inh1	%Inh2	mean	calc
50.0	56%	55%	56%	60%
16.67	34%	31%	33%	35%
5.56	30%	29%	29%	19%
1.852	18%	23%	21%	11%
0.617	10%	1%	5%	8%
0.2058	-16%	-13%	-14%	7%
0.0686	1%	9%	5%	7%
0.02286	16%	16%	16%	7%

4.3.5 Microsomal intrinsic clearance individual data

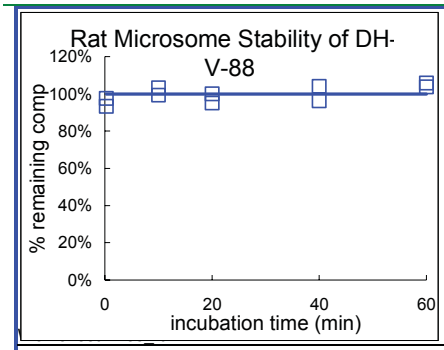


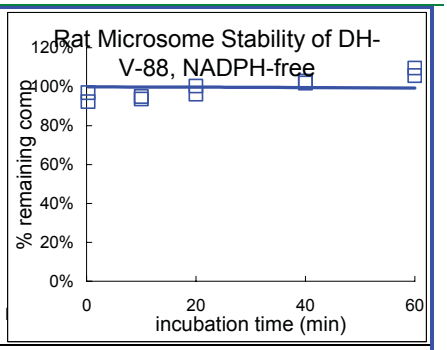


Test Agent: DH-V-88 **[TA] (uM):** 5.0

Species: Human

	W NADPH				W/O NADPH			
	CLint	0	**T1/2**	>180	**CLint**	0	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	87%	96%	91%	100%	97%	95%	96%	100%
10	98%	101%	100%	100%	97%	98%	98%	100%
20	94%	98%	96%	100%	99%	100%	100%	100%
40	105%	105%	105%	100%	103%	101%	102%	100%
60	107%	105%	106%	100%	103%	104%	104%	100%

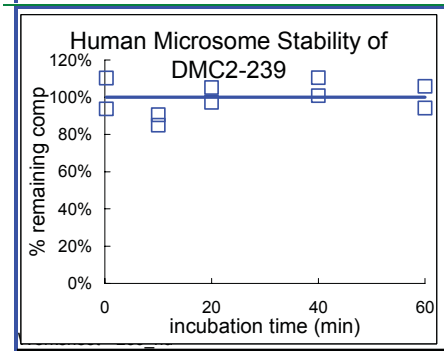


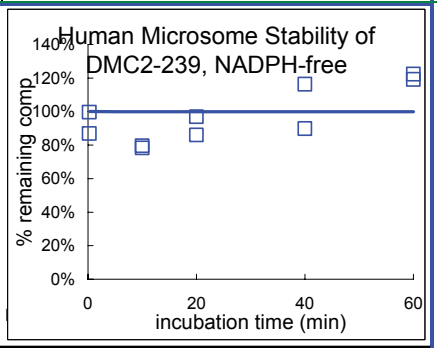


Test Agent: DH-V-88 **[TA] (uM):** 5.0

Species: Rat

	W NADPH				W/O NADPH			
	CLint	0	**T1/2**	>180	**CLint**	0	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	93%	98%	95%	100%	97%	92%	95%	100%
10	99%	103%	101%	100%	95%	94%	94%	100%
20	95%	100%	97%	100%	100%	96%	98%	100%
40	96%	104%	100%	100%	103%	102%	102%	100%
60	104%	106%	105%	100%	106%	110%	108%	99%

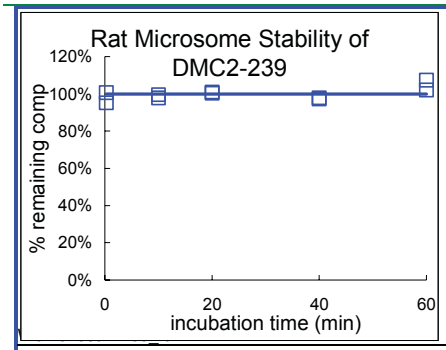


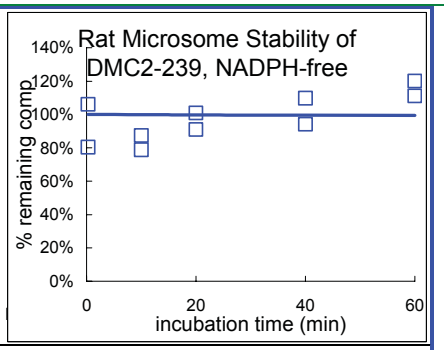


Test Agent: DMC2-239 **[TA] (uM):** 5.0

Species: Human

	W NADPH				W/O NADPH			
	CLint	0	**T1/2**	>180	**CLint**	0	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	110%	94%	102%	100%	87%	100%	93%	100%
10	85%	91%	88%	100%	79%	78%	79%	100%
20	97%	105%	101%	100%	86%	97%	91%	100%
40	110%	101%	106%	100%	90%	116%	103%	100%
60	106%	94%	100%	100%	119%	122%	121%	100%



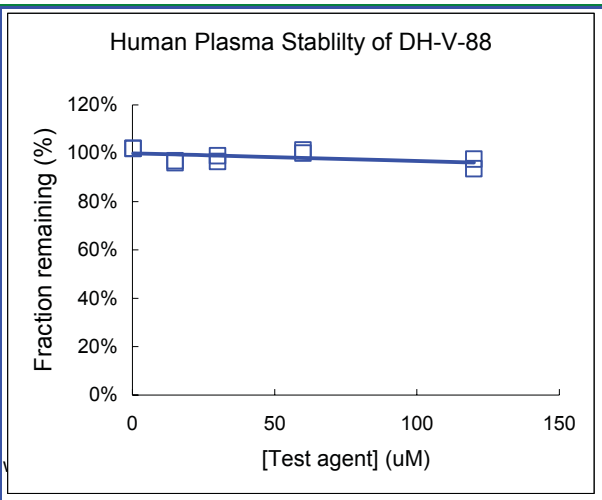


Test Agent: DMC2-239 **[TA] (uM):** 5.0

Species: Rat

	W NADPH				W/O NADPH			
	CLint	0	**T1/2**	>180	**CLint**	0	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	101%	95%	98%	100%	80%	106%	93%	100%
10	100%	98%	99%	100%	79%	87%	83%	100%
20	101%	100%	101%	100%	101%	91%	96%	100%
40	98%	97%	98%	100%	94%	110%	102%	100%
60	107%	102%	105%	100%	120%	111%	116%	99%

4.3.6 Plasma half-life individual data

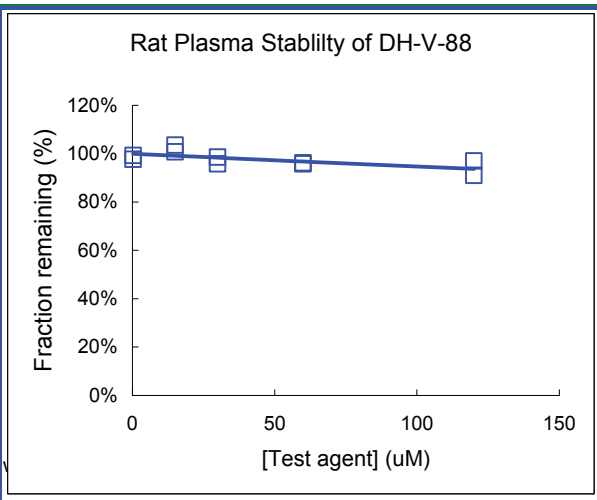


TA: DH-V-88 **Test conc (uM):** 10

Test medium: Human Plasma

T1/2 (min) >120 **%rem, last time** 95.6%

time	%RE1	%RE2	avg	calc
0.25	102%	102%	102%	100%
15	96%	97%	96%	100%
30	97%	99%	98%	99%
60	100%	101%	101%	98%
120	94%	98%	96%	96%

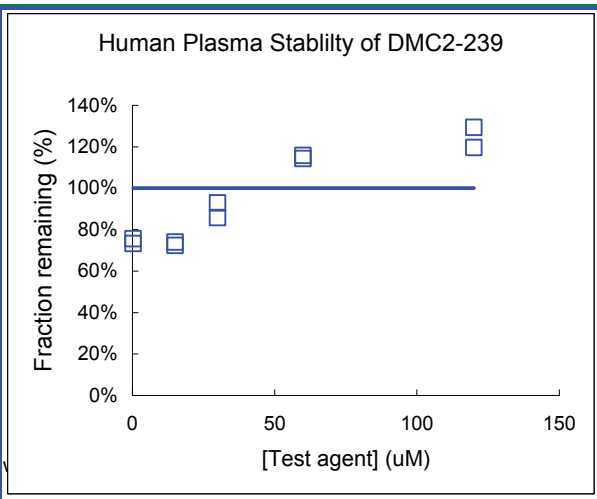


TA: DH-V-88 **Test conc (uM):** 10

Test medium: Rat Plasma

T1/2 (min) >120 **%rem, last time** 94.0%

time	%RE1	%RE2	avg	calc
0.25	98%	99%	98%	100%
15	103%	101%	102%	99%
30	99%	96%	97%	98%
60	96%	96%	96%	97%
120	97%	91%	94%	94%

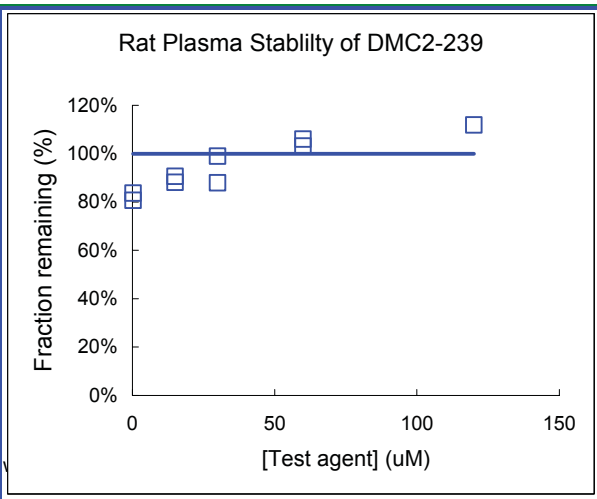


TA: DMC2-239 **Test conc (uM):** 10

Test medium: Human Plasma

T1/2 (min) >120 **%rem, last time** 124.5%

time	%RE1	%RE2	avg	calc
0.25	76%	73%	74%	100%
15	74%	72%	73%	100%
30	93%	86%	89%	100%
60	114%	116%	115%	100%
120	129%	120%	124%	100%



TA: DMC2-239 **Test conc (uM):** 10

Test medium: Rat Plasma

T1/2 (min) >120 **%rem, last time** 120.3%

time	%RE1	%RE2	avg	calc
0.25	81%	84%	82%	100%
15	91%	88%	89%	100%
30	99%	88%	93%	100%
60	106%	103%	105%	100%
120	129%	112%	120%	100%

4.3.7 Plasma protein binding individual data

			free fraction (%)			
Client ID	*test species*	*test conc (μM)*	*1st*	*2nd*	*mean*	*comment*
DH-V-88	Human	10	0.00000%	0.0030%	0.0015%	
DH-V-88	Rat	10	0.00114%	0.0020%	0.0016%	
DMC2-239	Human	10	0.00000%	0.0000%	0.0000%	
DMC2-239	Rat	10	0.00000%	0.0079%	0.0040%	

[a]peak area(analyte) / peak area (internal standard)

5 References

Stewart, BH, *et al*. (1995) "Comparison of intestinal permeabilities determined in multiple *in vitro* and *in situ* models: Relationship to absorption in humans." Pharm. Res. 12:693.

Crespi, CL, Stresser, DM (2000) "Fluorometric screening for metabolism-based drug-drug interactions." J. Pharmacol. Toxicol. Methods. 44:325.

Houston, JB (1994) "Utility of in vitro drug metabolism data in predicting in vivo metabolic clearance." Biochem. Pharmacol. 47:1469.

Banker, MJ, *et al*. (2003) "Development and validation of a 96-well equilibrium dialysis apparatus for measuring plasma protein binding." J. Pharm. Sci. 92:967.

6 Storage and Retention of Records

All documents generated in this study (raw data, the study plan, a copy of this report, etc.) will be stored for three years from the date of this document. Only authorized Apredica employees will have access to the archives.

The original final report will be provided to the sponsor and will be kept by the sponsor under its sole responsibility.

7 Appendices

7.1 Appendix A. Standard Apredica Methods

Caco-2 monolayer permeability

CaCo-2 cells grown in tissue culture flasks are trypsinized, suspended in medium, and the suspensions were applied to wells of a collagen-coated BioCoat Cell Environment in 24-well format (BD Biosciences) at 24,500 cells per well. The cells are allowed to grow and differentiate for three weeks, feeding at 2-day intervals.

For Apical to Basolateral (A->B) permeability, the test agent is added to the apical (A) side and amount of permeation is determined on the basolateral (B) side; for Basolateral to Apical (B>A) permeability, the test agent is added to the B side and the amount of permeation is determine on the A side. The A-side buffer contains 100 µM Lucifer yellow dye, in Transport Buffer (1.98 g/L glucose in 10 mM HEPES, 1x Hank's Balanced Salt Solution) pH 6.5, and the B-side buffer is Transport Buffer, pH 7.4. CaCo-2 cells are incubated with these buffers for 2 h., and the receiver side buffer is removed for analysis by LC/MS/MS.

To verify the CaCo-2 cell monolayers are properly formed, aliquots of the cell buffers are analyzed by fluorescence to determine the transport of the impermeable dye Lucifer Yellow.

Data are expressed as permeability (P_{app}): $P_{app} = \frac{dQ/dt}{C_0 A}$.

where dQ/dt is the rate of permeation, C_0 is the initial concentration of test agent, and A is the area of the monolayer.

In bidirectional permeability studies, the asymmetry index (AI) is also calculated:

$$\mathrm{AI} = \frac{P_{app}(B \to A)}{P_{app}(A \to B)}$$.

An AI > 1 indicated a potential substrate for PGP or other active transporters.

Cytotoxicity

HepG2 human hepatocellular carcinoma cells (originally obtained from ATCC, Manassas, VA) are seeded in 96-well plates at 10,000 cells per well, and grown for 24 hr in Eagle's Modified Essential Medium supplemented with 2 mM glutamine, nonessential amino acids, 2 mM pyruvate, 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin at 37ºC in 5% CO_2. NIH/3T3 mouse fibroblasts (originally obtained from ATCC) are seeded in 96-well plates at 10,000 cells per well, and grown for 24 hr in Dulbecco's Modified Essential Medium supplemented with 10% bovine calf serum, 100 U/mL penicillin, and 100 mg/L streptomycin at 37ºC in 5% CO_2. . HaCaT human keratinocytes are seeded in 96-well plates at 10,000 cells per well, and grown for 24 hr in Dulbecco's Modified Essential Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin at 37ºC in 5% CO_2. .

Test article is aseptically diluted in DMSO to 200x the highest concentration, then 100-fold in the growth medium, and serial dilutions are made in 1% DMSO in growth medium. At the start of the assay, the growth medium is removed from the plates and replaced with fresh medium, and an equal volume from each test agent dilution is added. Cells are incubated with test article for 48 h, and the wells are examined microscopically to look for abnormalities.

For Neutral red staining, medium is removed, the cells are washed with PBS, and fresh medium containing 25 μg/mL neutral red (Sigma) is added. After four hours incubation, the cells are washed with PBS, and the cellular dye is solubilized with 1% acetic acid in 50% ethanol. Cellular neutral red is measured by its absorbance at 540 nm. Cytotoxicity is assessed by determining the IC_{50} (the concentration that causes 50% reduction in uptake of neutral red after 48 hrs exposure to compound.

For MTT staining, 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT, 5 mg/mL in PBS, Sigma), is added to each well. After two hours incubation, the medium is removed, and the cellular dye is solubilized with DMSO. Cellular-converted MTT is measured by its absorbance at 540 nm. Cytotoxicity is assessed by determining the IC50 (the concentration that causes 50% reduction in uptake of MTT after 48 hrs exposure to compound).

Fluorescent cytochrome P450 IC_{50} determination

Cytochrome P450 inhibition is measured using fluorogenic substrates. Test agents and substrates are dissolved in acetonitrile for this assay, as DMSO significantly inhibits some cytochrome P450s. Assays were performed at 37 °C using commercially available recombinant human cytochrome P450 expressed in insect cells. Enzyme concentrations and reactions times are optimized for each batch of enzyme to ensure a linear production of product over the course of the reaction. Percent remaining activity is calculated by comparing product formation of wells treated with test agent against wells treated with vehicle, after subtraction of background fluorescence. Percent inhibition is 100% - percent remaining activity. IC_{50} is calculated using a four-point logistic curve model. The individual reaction conditions are summarized in the following Table.

Cytochrome	Substrate	Assay Buffer
1A2	5 μM CEC	Buffer B
2A6	3 μM coumarin	Buffer D
2B6	15 μM MFC	Buffer B
2C8	1 μM DBF	Buffer E
2C9	1 μM DBF	Buffer A
2C19	2 μM DBF	Buffer B
2D6	1.5 μM AMMC	Buffer C
2E1	100 μM MFC	Buffer B
3A4	50 μM BFC	Buffer B
3A4	1 μM DBF	Buffer B
3A5	50 μM BFC	Buffer B
Aromatase	0.5 μM DBF	Buffer B

Buffer A: 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 25 mM potassium phosphate, 3.3 mM $MgCl_2$, pH 7.4.
Buffer B: 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 100 mM potassium phosphate, 3.3 mM $MgCl_2$, pH 7.4.
Buffer C: 8.2 μM NADP, 0.41 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 100 mM potassium phosphate, 0.41 mM $MgCl_2$, pH 7.4.
Buffer D: 0.066 mM NADP, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 100 mM Tris hydrochloride, 3.3 mM $MgCl_2$, pH 7.5.
Buffer E: 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 50 mM potassium phosphate, 3.3 mM $MgCl_2$, pH 7.4.
Abbreviations: AMMC: 3-[2-(N,N-Diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin; BFC:7-Benzyloxy-4-(trifluoromethyl)couamrin CEC: 3-Cyano-7-ethoxycoumarin; DBF: dibenzylfluorescein; MFC: 7-Methoxy-4-(trifluoromethyl)coumarin

hERG FastPatch

Cell culture. HEK293 cells were stably transfected with hERG cDNA. Stable

transfectants have been selected by coexpression with the G418-resistance gene incorporated into the expression plasmid. Selection pressure was maintained by including G418 in the culture medium. Cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/ F-12) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulfate and 500 µg/mL G418. Before testing, cells in culture dishes were washed twice with Hank's Balanced Salt Solution, treated with trypsin and re-suspended in the culture media (1-1.5 $x10^6$ cells in 20 mL). Cells in suspension were allowed to recover for 1-3 hours in a tissue culture incubator set at 37°C in a humidified 95% air, 5% CO2 atmosphere. Immediately before use in the PatchXpress® system, the cells were washed in HB-PS to remove the culture medium and re-suspended in 150 µL of HB-PS.

Test Method. All experiments were performed at ambient temperature. Each cell acted as its own control.

Test Article Treatment Groups. Two concentrations were applied at five (5) minute intervals via disposable polyethylene micropipette tips to cells expressing hERG (n ≥ 2, where n = the number cells/concentration). Each solution exchange, performed in quadruplicate, consisted of aspiration and replacement of 45 µL of the total 50 µL volume of the extracellular well of the Sealchip16. Duration of exposure to each test article concentration was five (5) minutes.

Test Article Application Schedule.

Solution	Procedure	Exposure time
Vehicle control	four 45 µL exchanges	10 min
Test article concentration 1	four 45 µL exchanges	5 min
Test article concentration 2	four 45 µL exchanges	5 min

Positive Control Treatment Group. Vehicle was applied to cells expressing hERG (n ≥ 2, where n = the number cells), for a 10-minute exposure interval. Each solution exchange, performed in quadruplicate, consisted of aspiration and replacement of 45 µL of the total 50 µL volume of the extracellular well of the Sealchip16. After vehicle application, the positive control was applied in the same manner, to verify sensitivity to hERG blockade.

Automated Patch Clamp Electrophysiological Procedures. Intracellular solution for whole cell recordings consisted of (composition in mM): potassium aspartate, 130; MgCl2, 5; EGTA, 5; ATP, 4; HEPES, 10; pH adjusted to 7.2 with KOH. This solution was prepared in batches, aliquoted, stored frozen, and a fresh aliquot thawed each day. In preparation for a recording session, intracellular solution was loaded into the intracellular compartments of the Sealchip16 planar electrode. Cell suspension was pipetted into the extracellular compartments of the Sealchip16 planar electrode. After establishment of a whole-cell configuration, membrane currents were recorded using dual-channel patch clamp amplifiers in the PatchXpress® system. Before digitization, the current records were low-pass filtered at one-fifth of the sampling frequency.

Voltage-Clamp Procedures. Onset and block of hERG current was measured using a stimulus voltage pattern (Figure 1, lower panel) consisting of a 500 ms prepulse to –40 mV (leakage subtraction), a 2-second activating pulse to +40 mV, followed by a 2-second test pulse to -40 mV. The pulse pattern was repeated continuously at 10 s intervals, from a holding potential of -80 mV. Peak tail current (Figure 1, upper panel) was measured during the -40 mV test pulse. Leakage current was calculated from the current amplitude evoked by the prepulse and subtracted from the total membrane current record.

Microsomal intrinsic clearance

The test agent is incubated in duplicate with microsomes at 37 ºC. The reaction contains microsomal protein in 100 mM potassium phosphate, 2 mM NADPH, 3 mM $MgCl_2$, pH 7.4. A control is run for each test agent omitting NADPH to detect NADPH-free degradation. The indicated times, an aliquot is removed from each experimental and

control reaction and mixed with an equal volume of ice-cold Stop Solution (0.3% acetic acid in acetonitrile containing haloperidol, diclofenac, or other internal standard). Stopped reactions are incubated at least ten minutes at -20 ºC, and an additional volume of water is added. The samples are centrifuged to remove precipitated protein, and the supernatants are analyzed by LC/MS/MS to quantitate the remaining parent.
Data are converted to % remaining by dividing by the time zero concentration value. Data are fit to a first-order decay model to determine half-life. Intrinsic clearance is calculated from the half-life and the protein concentrations: $CL_{int} = \ln(2) / (T_{1/2}$ [microsomal protein]*)*.

Plasma protein binding

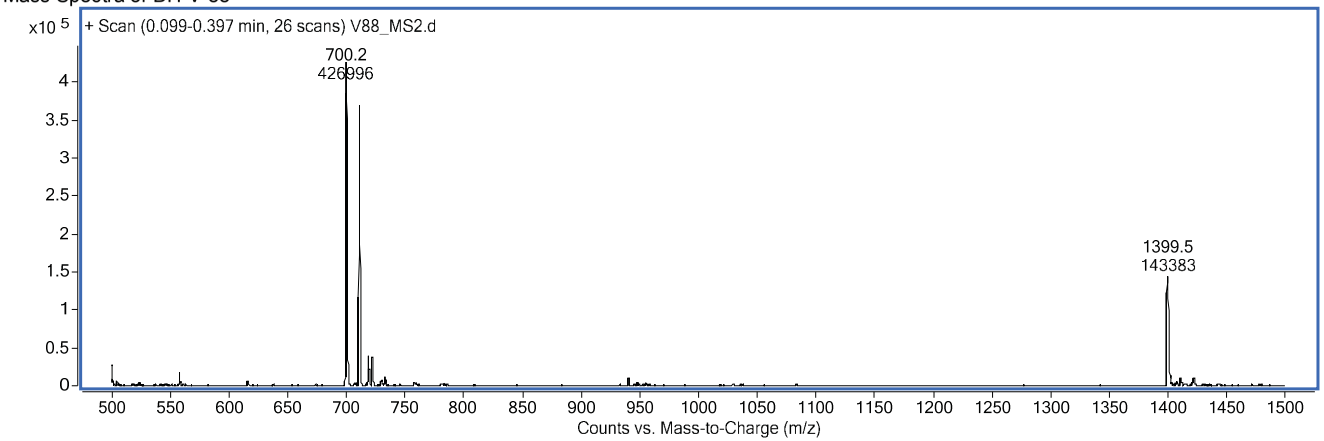
Test agent is added to plasma. This mixture is dialyzed in a RED Device (Pierce) per the manufacturers' instructions along against PBS and incubated in a rocker. After the end of the incubation, aliquots from both plasma and PBS sides are collected, an equal amount of PBS is added to the plasma sample, and an equal volume of plasma is added to the PBS sample. Methanol (three volumes) with haloperidol IS are added to precipitate the proteins and release the agents. After centrifugation, the supernatant was transferred to a new plate and analyzed by LC/MS/MS.

Plasma half-life

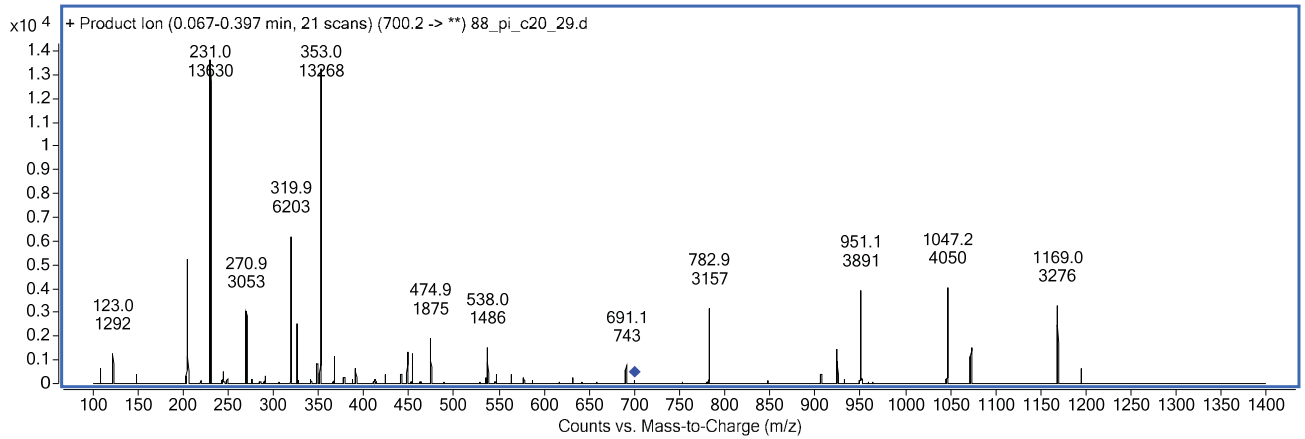
The test agent is incubated in duplicate with plasma at 37 ºC. The reaction contains plasma and 2% DMSO. At the indicated times, an aliquot is removed from each experimental reaction and mixed with three volumes of ice-cold Stop Solution (methanol containing propranolol, diclofenac, or other internal standard). Stopped reactions are incubated at least ten minutes at -20 ºC. The samples are centrifuged to remove precipitated protein, and the supernatants are analyzed by LC/MS/MS to quantitate the remaining parent. Data are converted to % remaining by dividing by the time zero concentration value. Data are fit to a first-order decay model to determine half-life.

7.2 Appendix B. Sample Spectra and Chromatograms of the Test Agents

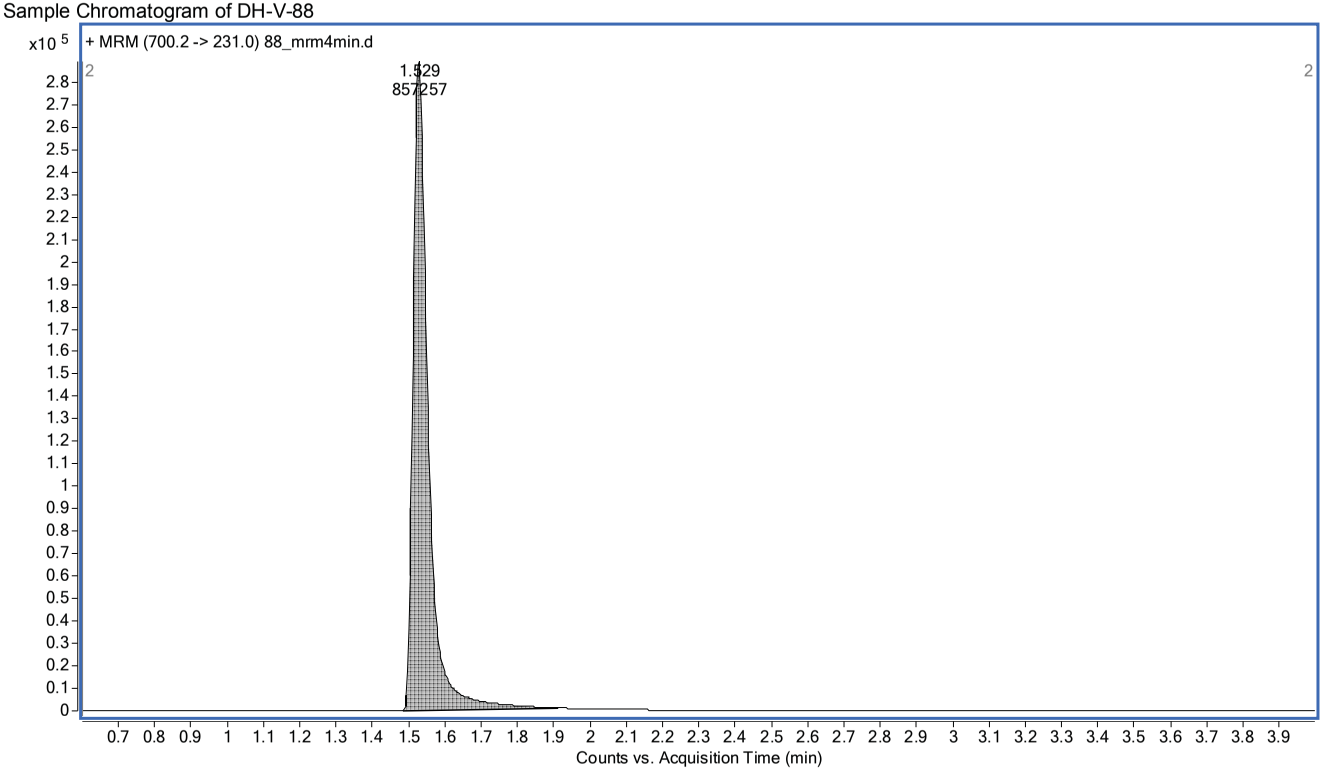
Mass Spectra of DH-V-88



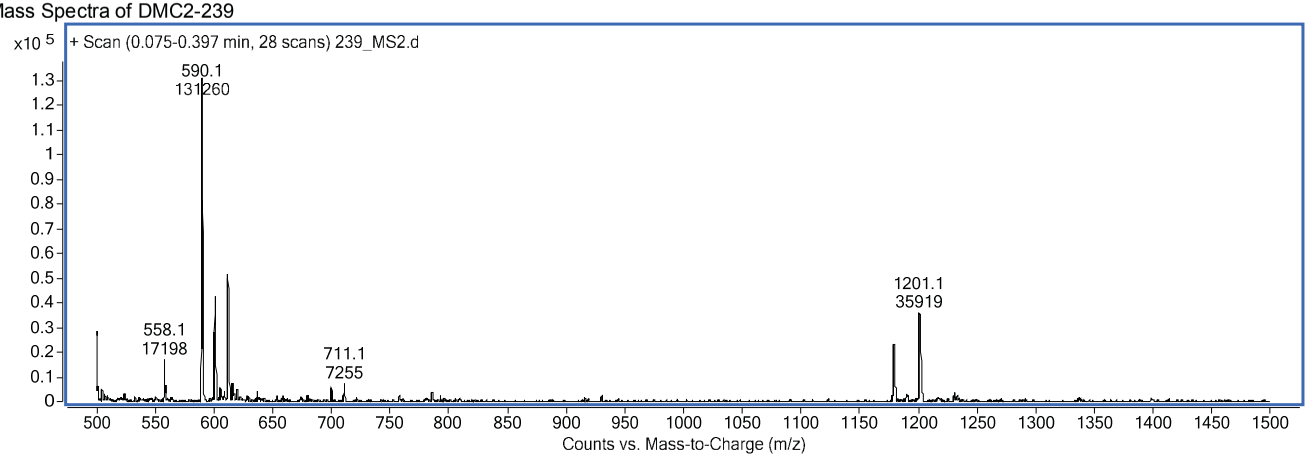
Product ion scan of DH-V-88



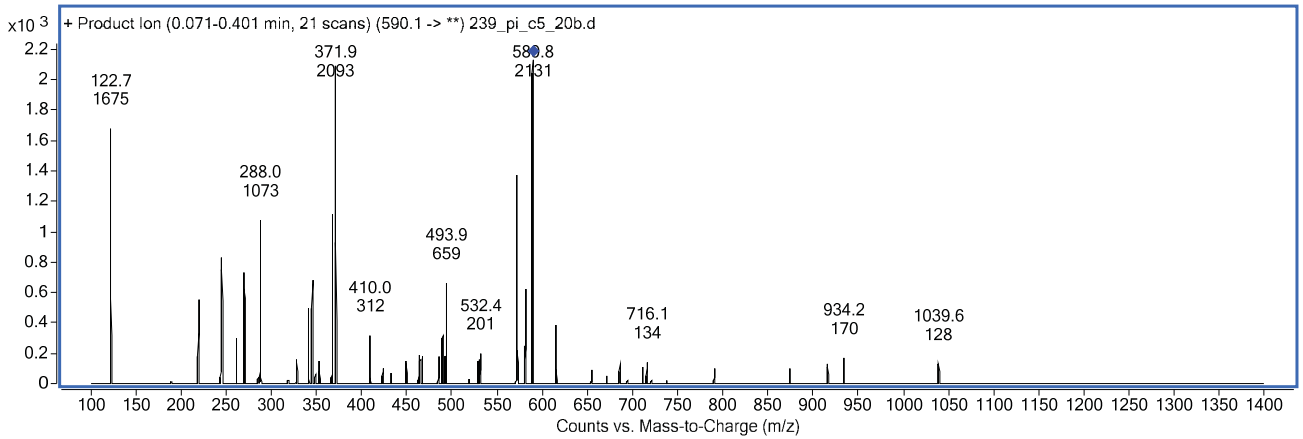
Sample Chromatogram of DH-V-88



Mass Spectra of DMC2-239



Product ion scan of DMC2-239



Sample Chromatogram of DMC2-239