The Use of Modified Bacterial CYPs for Metabolite Generation

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SCDMDG meeting
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VERTEX

Objectives

- Prepare mg quantities of human metabolites of compounds and study their activity (use as bioanalytical standards)

- Prepare non-human metabolites (‘unnatural metabolites’) for lead diversification (with improved properties)

- Investigate unnatural metabolites of known drugs and/or failed drugs as an approach to obtaining novel drug-like entities

- Scale-up of and purification of metabolites for more rapid in vitro profiling

- Identification and production of new mutants which metabolize a wider variety of compounds (>80%)

BM3 Mutant Cytochrome P450s are Used for Metabolite Preparation/Isolation

BM3 P450 has better potential for metabolite preparation than mammalian microsomal CYPs

Advantages:

- ➔ *Stable, water soluble*
- ➔ *Inexpensive*
- ➔ *Rapid turn-over rate*
- ➔ *Diversity of metabolites produced for SAR studies (see later)*

Disadvantage or advantage:

- ➔ Substrate specificity different from that of mammalian CYPs, natural substrates are fatty acids

Background: Creation of BM3 mutants

Wild-type BM3

Bacillus megaterium reductase and heme fused

Site-directed mutagenesis

BM3 triple mutant

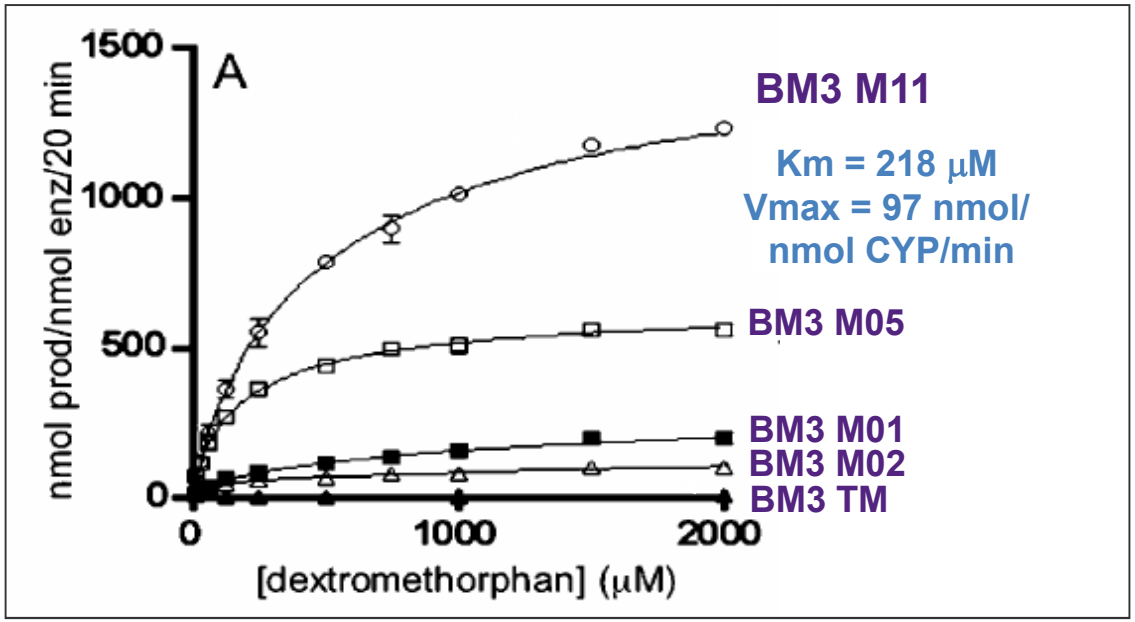
Error-prone PCR

BM3-MXX

Random mutant library

M01, M02, … M11…

Screen P450 for activity



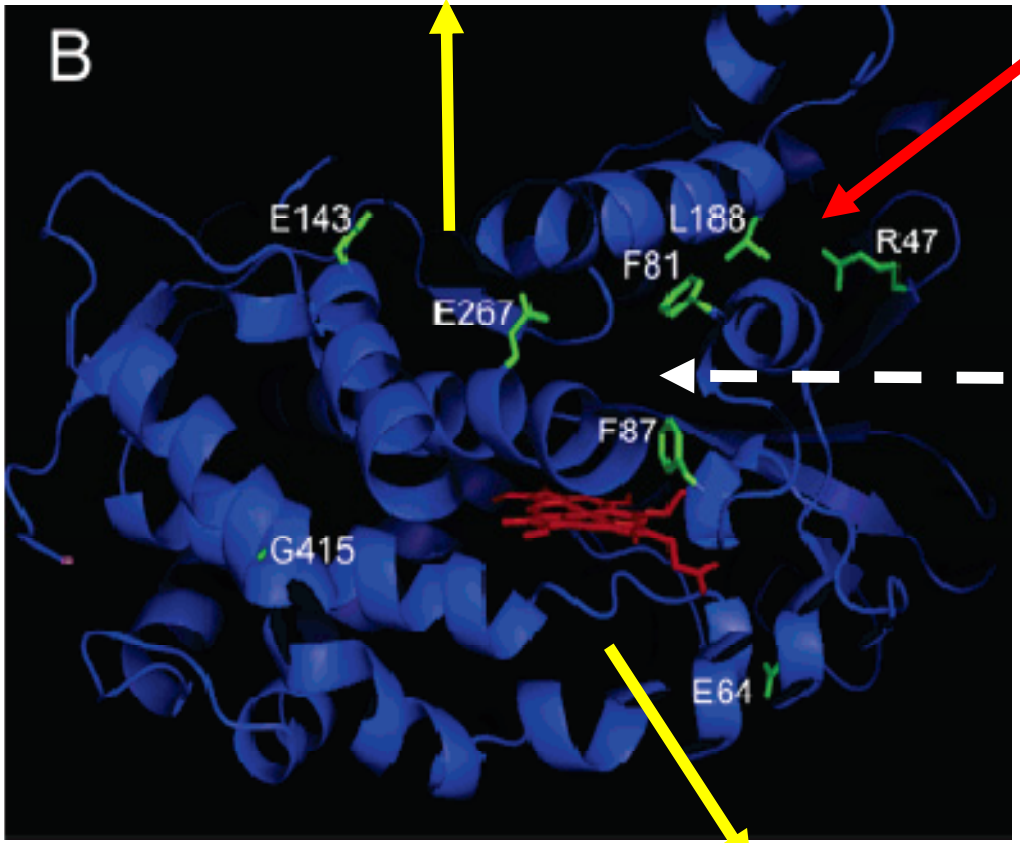
Metabolite: N-demethylated dextromethorphan

Dextromethophan N-demethylation of BM3-M11 is 900 X that of the BM3TM (triple mutant)

Reference: N. P.E. Vermeulen, J. Commandeur, et. al, "*Identification of Critical Residues in Novel Drug Metabolizing Mutants of Cytochrome P450 BM3 Using Random Mutagenesis.*" J. Med. Chem., **2007**, 50, 455-461.

BM3 M11 - Rationalizing the Effects of Random Mutagenesis

Putative product exit channel (ii)
***E267V**: close exit **E143G**: open exit*



Putative substrate access channel
***F81V**: opening up access for bulkier substrates*
***L188Q & R47L**: maintain polar environment*

Putative active site area
***F87V:** opening up active site for bulkier substrates [present in all mutants]*
***F81I**: shape change hydrophobic pocket*

***G415S**: shown to not contribute to enhanced mutant activity*

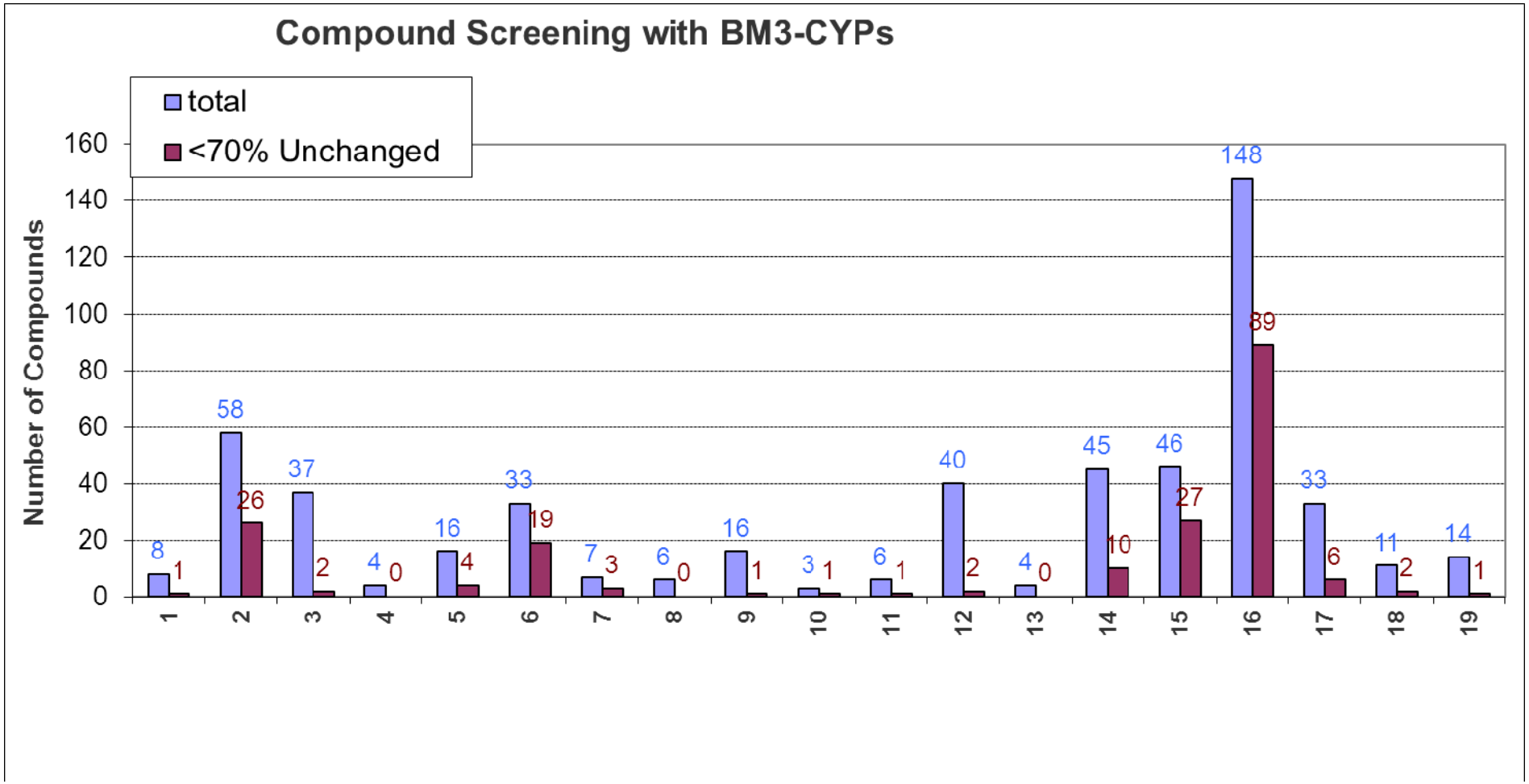
Putative product exit channel (i)
***E64G**: facilitate exit*

Reference: N. P.E. Vermeulen, J. Commandeur, et. al, "*Identification of Critical Residues in Novel Drug Metabolizing Mutants of Cytochrome P450 BM3 Using Random Mutagenesis.*" J. Med. Chem., **2007**, 50, 455-461.

Fermentation Procedures Established in House

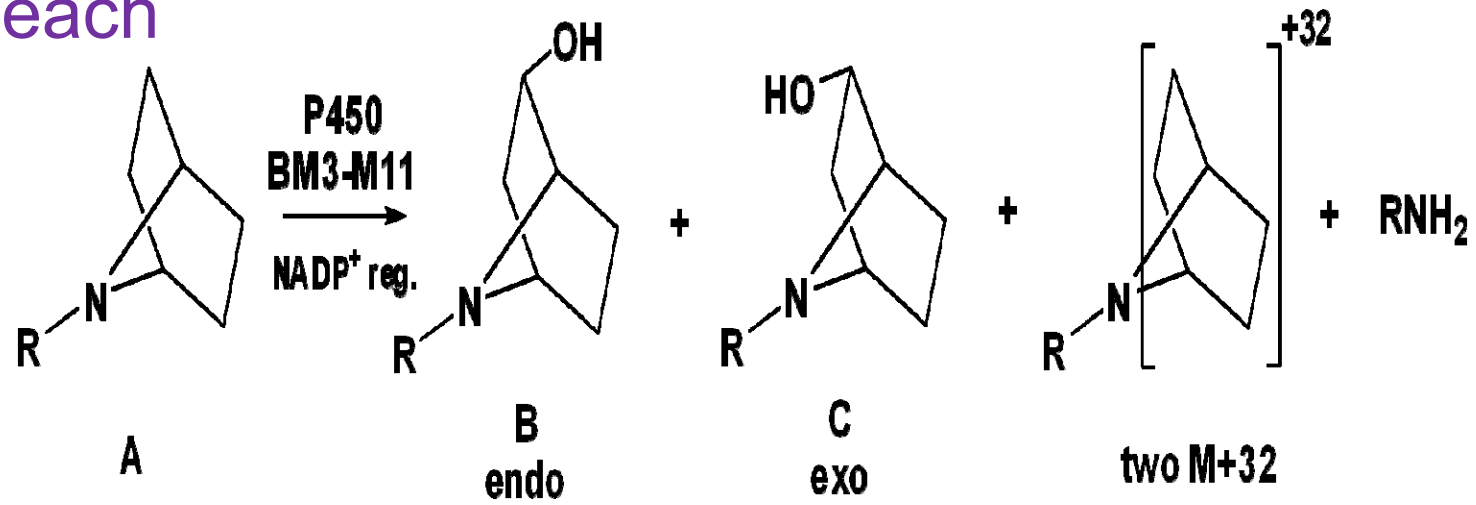
Approximately 700 Compounds Screened

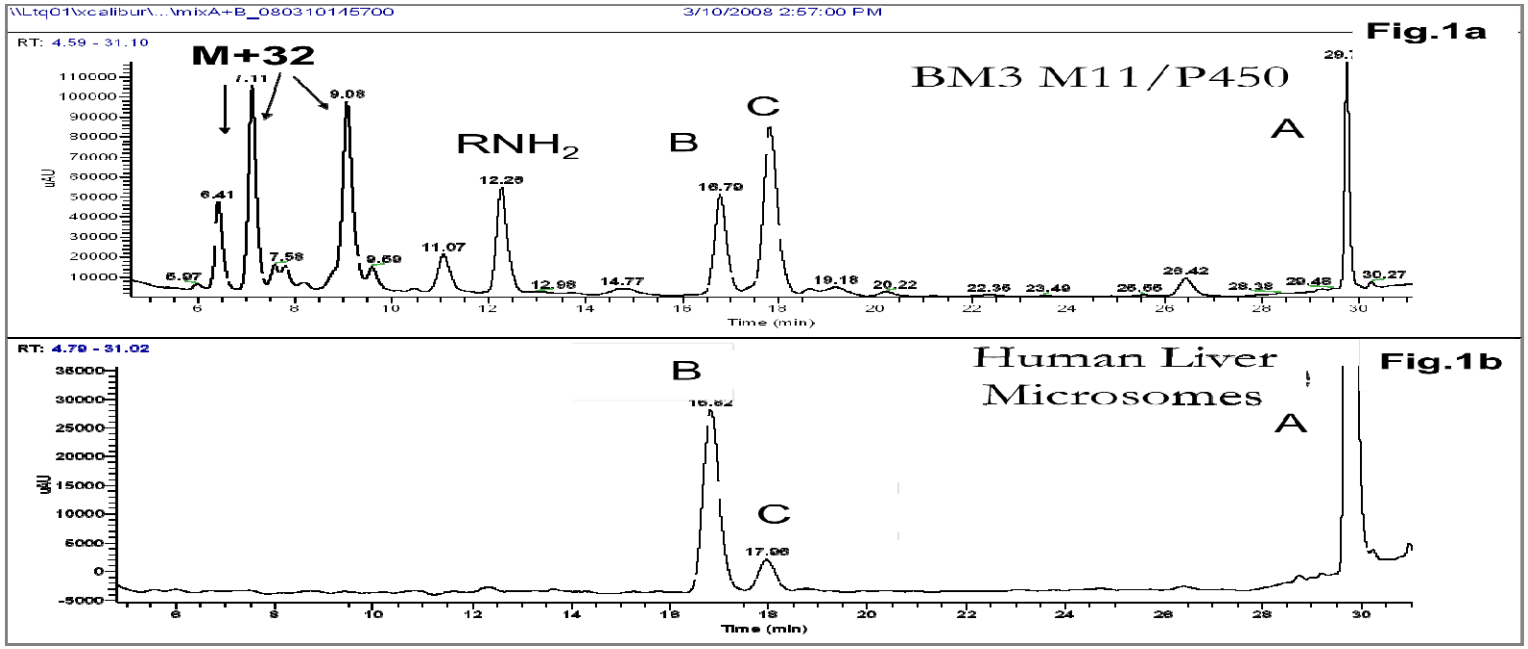
(36% of all compounds tested are metabolized by BM3-P450s at >30%)



Case Study 1: Sovent Effects upon and Enantiomeric Selectivity of BM3-M11 in the Metabolism of Compound A

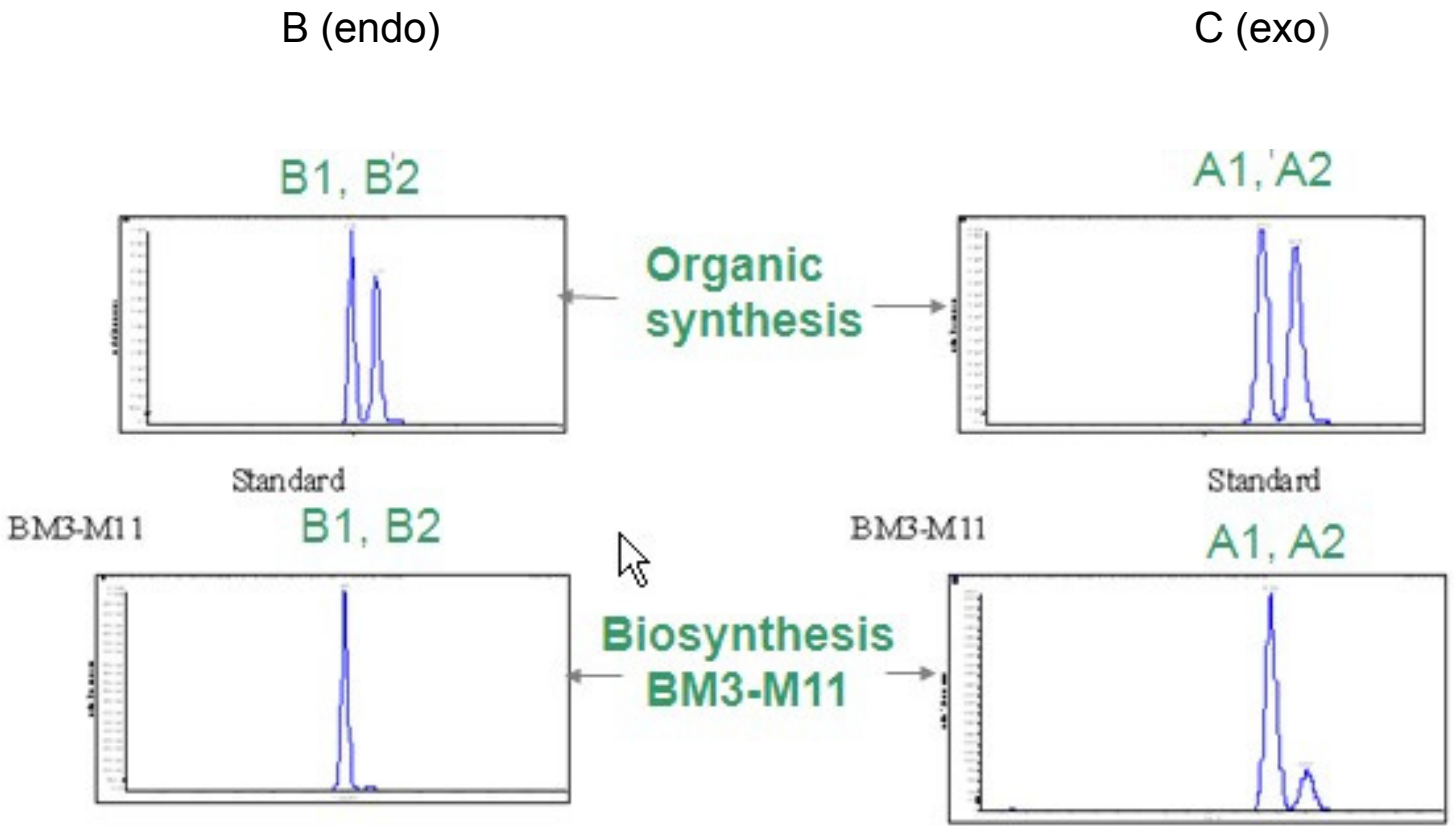
BM3-M11 Produces 5 Metabolites of Compound A, 1 to 2 mg/each





L.Sun et al,
ISSX proceedings
Istanbul 2010

B and C Metabolites are Chiral



- BM3-M11 oxidation is stereoselective

Metabolite Profiling of Compound A in Liver Microsomes from 4 species

Metabolism of Compound A in Liver Microsomes
Species % peak area to parent

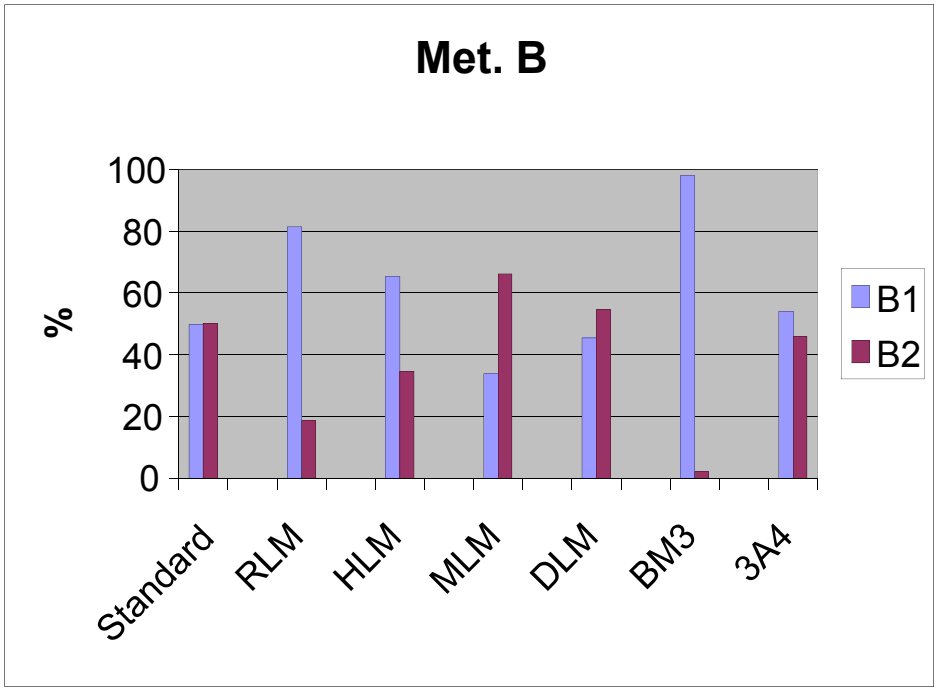
	B	C
Human	3.9	0.8
Monkey	3.5	0.5
Dog	0.9	1.1
Rat	6.5	0.6

0.5 mg/mL protein, 10 mMCompound A, 30 minute incubation, NAPPH 1 mM

- Relative abundances of the 2 hydroxylated metabolites vary between species

Enantiomer Ratios for Metabolite B (exo)

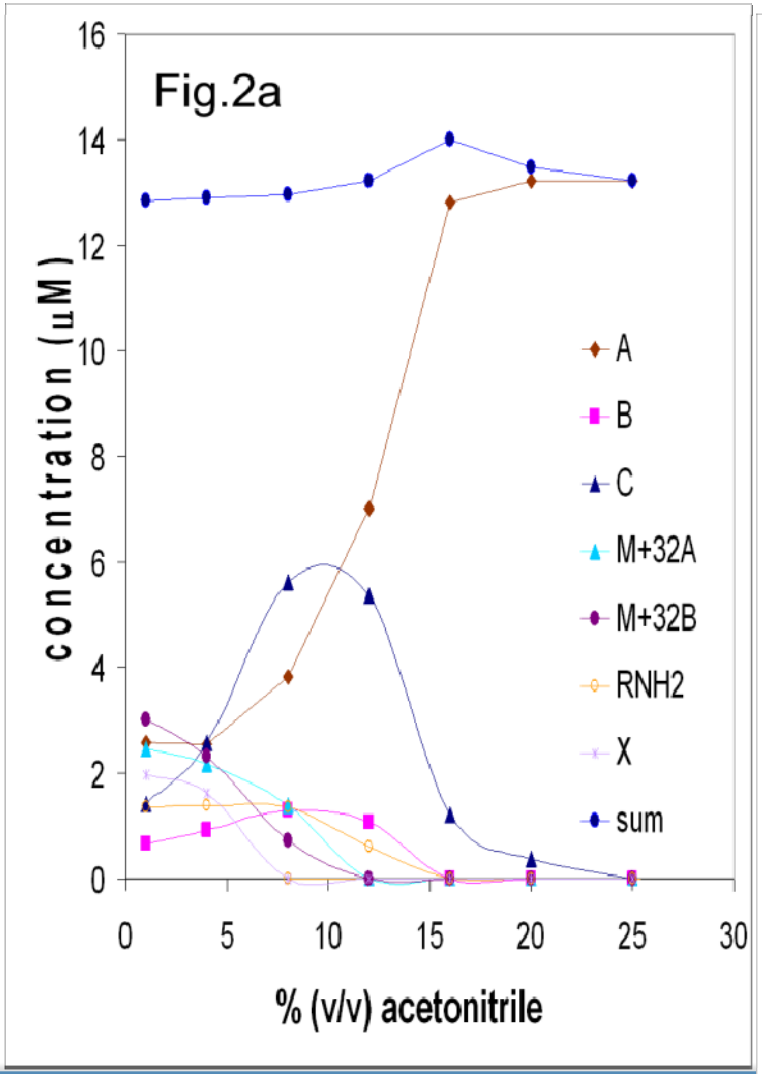
	Peak area ratio(B1/B2)	
	B1	**B2**
Standard	49.76	50.24
RLM	81.36	18.64
HLM	65.36	34.64
MLM	33.87	66.13
DLM	45.36	54.64
BM3	97.96	2.04
3A4	54.05	45.95

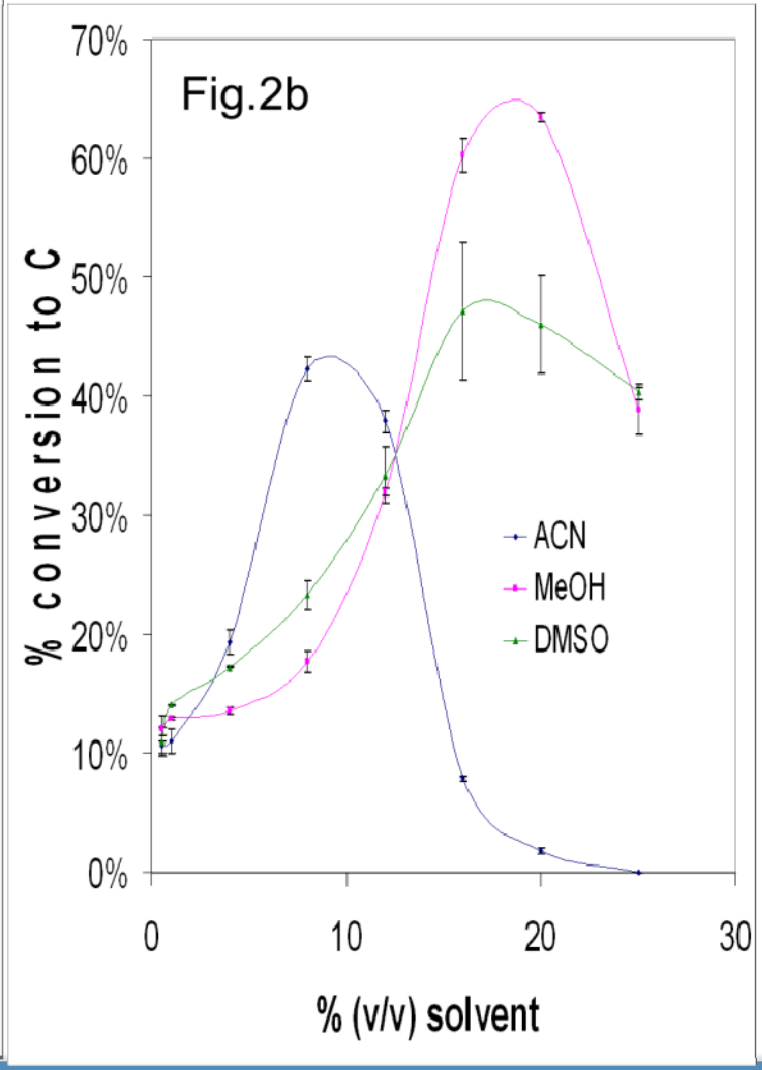


Ratio of B2 to B1 ~ 8.5 in dogs orally administered 10 mg/kg Compound A

Enantiomers of both B and C observed in vivo although at different ratios than in microsomes

Formation of A and B Metabolites from Compound A using Different ACN Concentrations and Comparison of Compound C Formation from Compound A using ACN, MeOH or DMSO





Conclusions

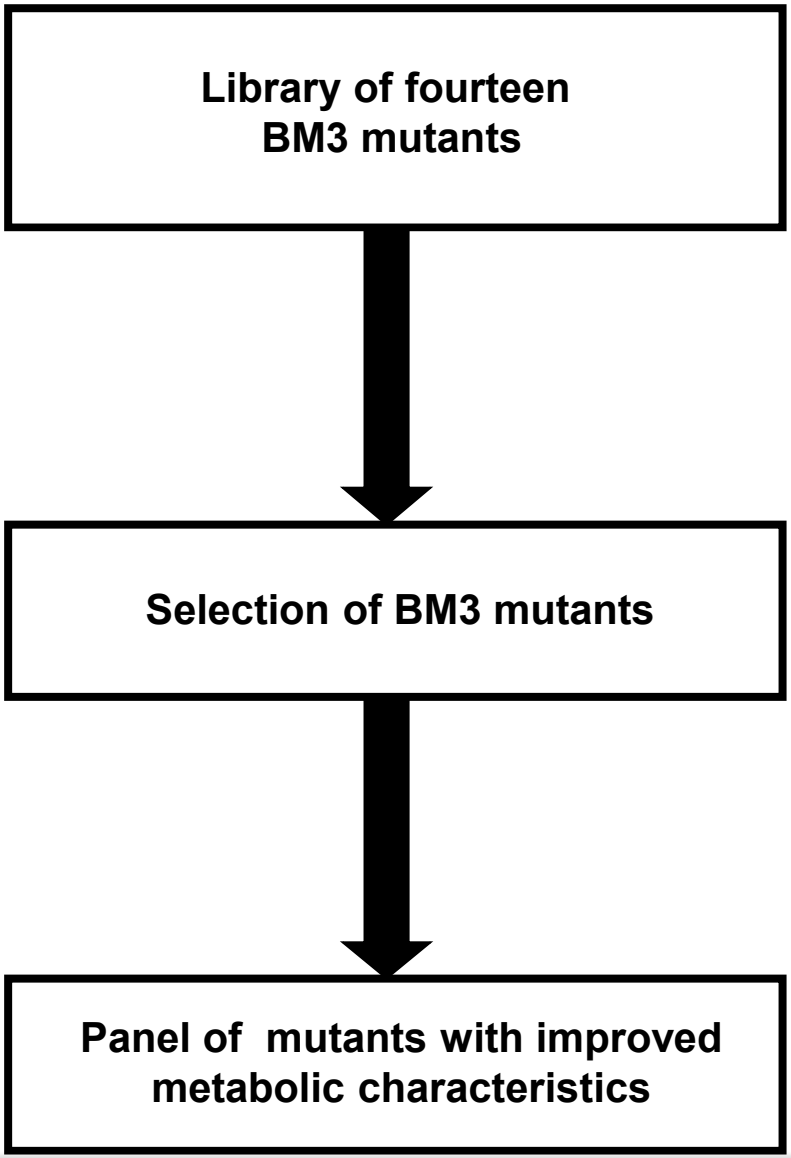
When the concentration of ACN increases, the formation of B and C from Compound A are increased and the formation of secondary metabolites decreases

The formation of compound C is enhanced by solvent addition (~20% MeOH, 17% DMSO, 10% ACN maximal). Thus, solvent addition can modify pathway preference

Of all solvents evaluated, MeOH > DMSO > ACN in enhancing Compound C formation

Case Study 2: Mutant Comparisons in Activity and Metabolite Selectivity for 43 Drugs

Research Strategy



Selection criteria (9 drugs):

- Metabolic activity
- Metabolic profiles
- Substrate diversity
- Coupling efficiencies

Selection criteria (43 drugs):

- Metabolic activity
- Metabolic profiles
- Substrate diversity

Reinen J. et al, DMD 39: 156801576 (2011)

Selection Criteria for 14 Screening Mutants

- Based upon the experiments performed a selection of 14 BM3 mutants was made against which the total set of drugs would be screened

  - Mutants which displayed the highest overall activities and coupling efficiencies et al., have previously described to exhibit good activity (van Vugt-Lussenburg et al. Med Chem 50:455-461 2007)

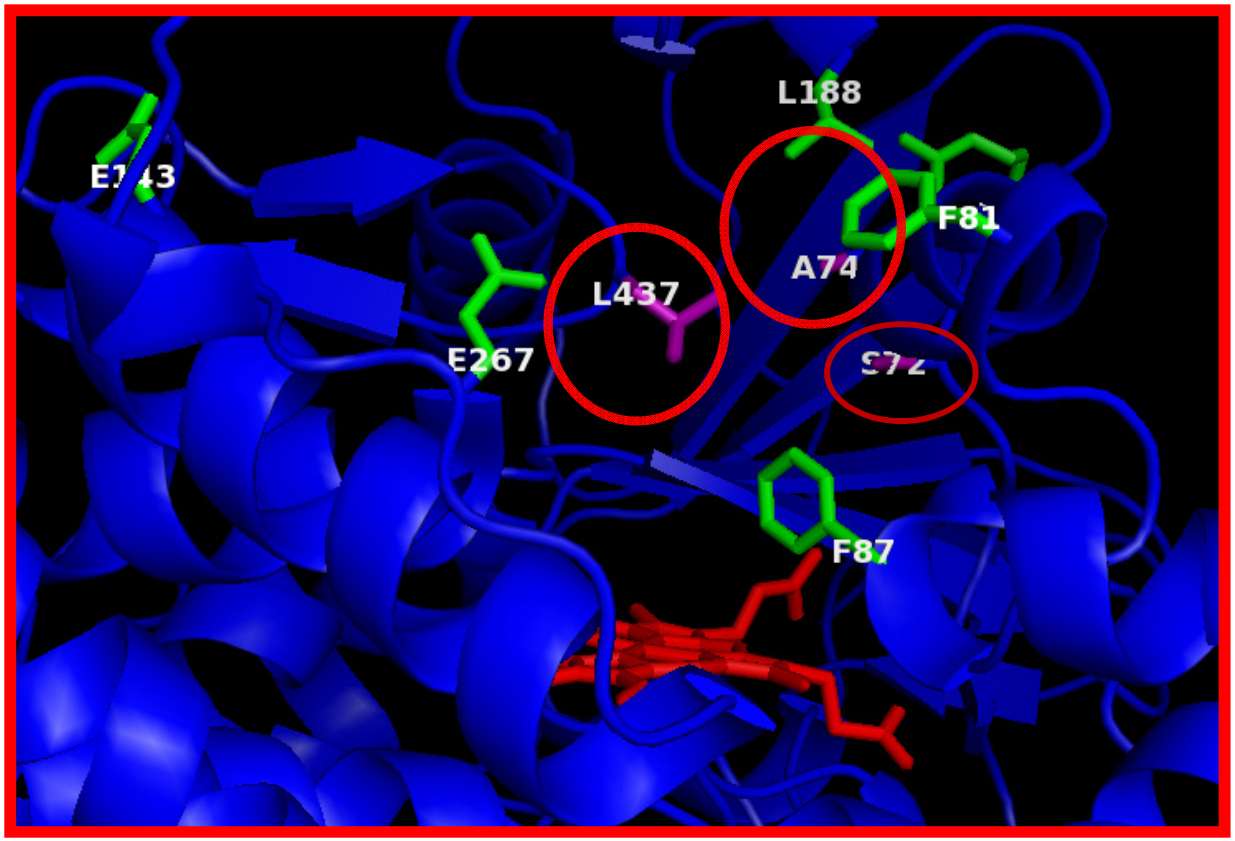
  - Mutants which displayed the highest overall activities and coupling efficiencies

  - 4 mutants of the Leu437 (involved in substrate contact) (Li H.and Poulos T. Nat Struct Biol 4:140-146 1997)

  - MT32 contained an extra negative charge in the active site at the 437 position (Leu)

  - MT33, MT35 and MT36 and MT36 contained an extra negative charge in the active site at the 437 position (Leu)

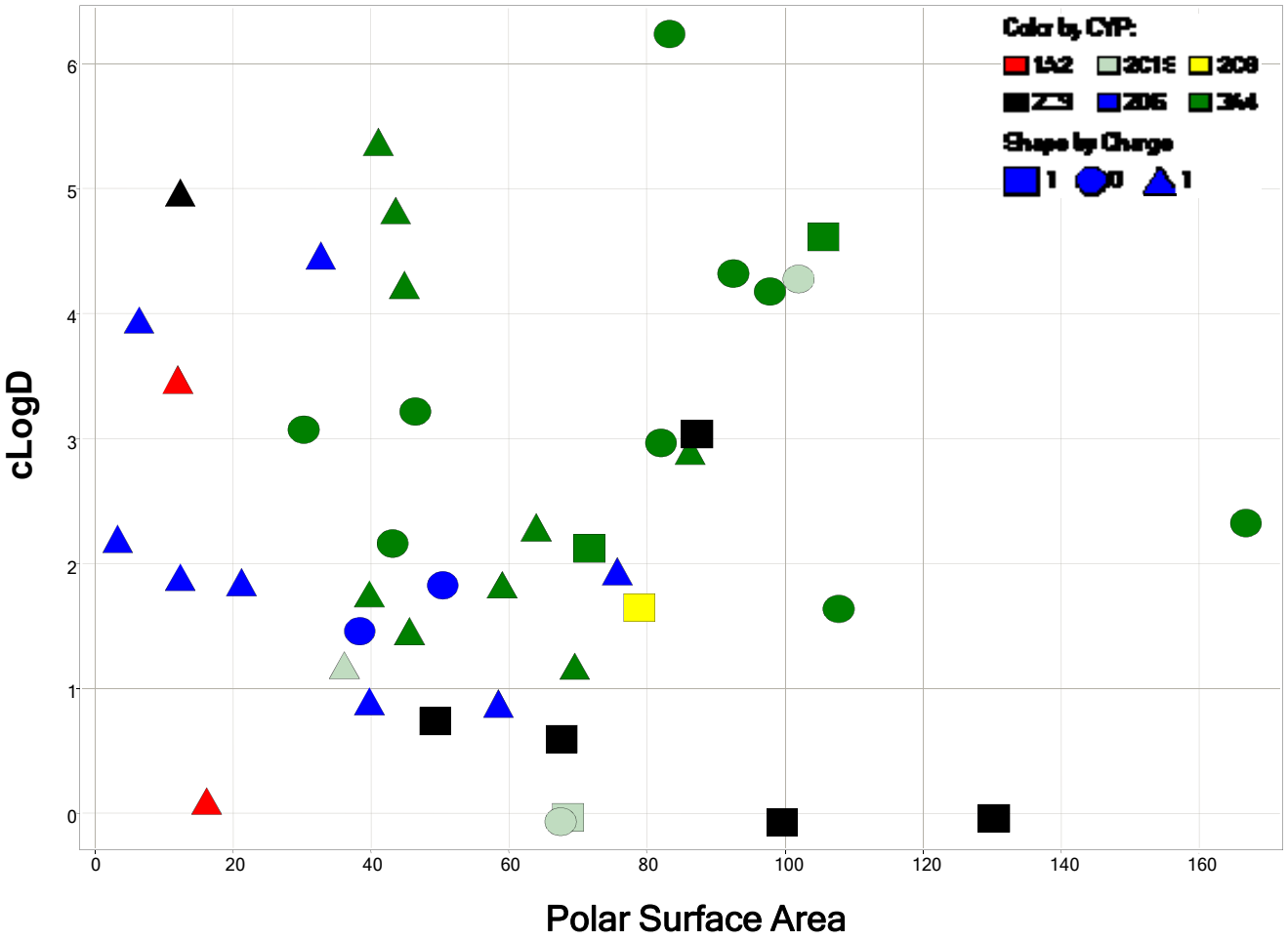
Location of the Different Mutations

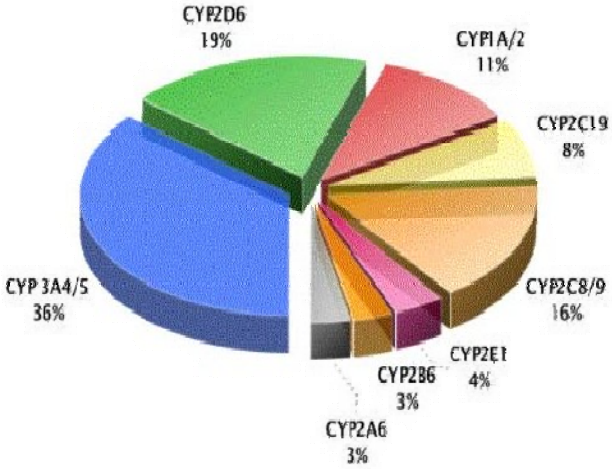


Modifications at L437, S72 and A74 are in active site

Crystal structure of the active site of P450 BM3 (PDB 1D 1BU7). The residues that were altered in mutant M11 are labeled by their amino acid numbers and depicted in green. The additional residues which have been altered in this study to obtain the different novel BM3 mutants are also labeled and are depicted in purple. The heme is displayed in red.

Selection Criteria for 43 Drugs Selected for Evaluation





The selected drug screening set spans a wide range of

- **Charges**
- **CYP phenotypes**
- **cLogD values**
- **size and MW**

43 Drug Screening Strategy

- Incubate all enzymes at a 500 nM enzyme concentration in the presence of 40 μM of substrate for 90 min
- The Viva tool in combination with LC/MS/MS was used to rapidly to determine substrate depletion levels and screen for selected MRMs for all 43 drugs
- Perform automated peak detection and integration for parent drugs and selected metabolites using Auto-Quan software
- Review data qualitatively using in-house developed software
- Use data to assess correlations of metabolism by the different BM3 mutants with the different properties of the 43 drugs

Substrate Depletion Results for Metabolism of Selected Drugs by 6 BM3 Mutants

Compound	M01[a]	M02[a]	M11[a]	MT35[a]	MT38[a]	MT43[a]	HLM
Amitriptyline	**36**	**34**	**74**	**96**	**84**	**25**	**18**
Aripiprazole	**99**	**99**	**99**	**93**	**99**	**99**	**93**
Astemizole	**95**	**8**	**94**	**68**	**<5 [b]**	**10**	**22**
Buspirone	**46**	**75**	**83**	**67**	**9**	**29**	**17**
Carbamazepine	**<5 [b]**	**<5 [b]**	**<5 [c]**	**11**	**<5 [b]**	**10**	**<5 [b]**
Carvedilol	**86**	**9**	**78**	**66**	**51**	**32**	**8**
Cilostazol	<5 [b]	25	17	37	82	93	38
Cinacalcet	**96**	**89**	**97**	**87**	**67**	**96**	**46**
Citalopram	**29**	**7**	**56**	**78**	**27**	**10**	**<5 [b]**
Dextromethorphan	**68**	**23**	**95**	**97**	**80**	**50**	**27**
Diltiazem	6	<5 [b]	<5 [b]	55	11	10	28
Duloxetine	18	<5 [b]	39	51	53	20	<5 [b]
Gleevec	**51**	**80**	**77**	**30**	**13**	**<5 [b]**	**<5 [b]**
Glipizide	**<5 [b]**	**<5 [b]**	**7**	**35**	**<5 [b]**	**9**	**44**
Irbesartan	12	<5 [b]	7	80	23	6	<5 [b]
Midazolam	<5 [c]	<5 [c]	<5 [c]	40	<5 [c]	<5 [c]	36

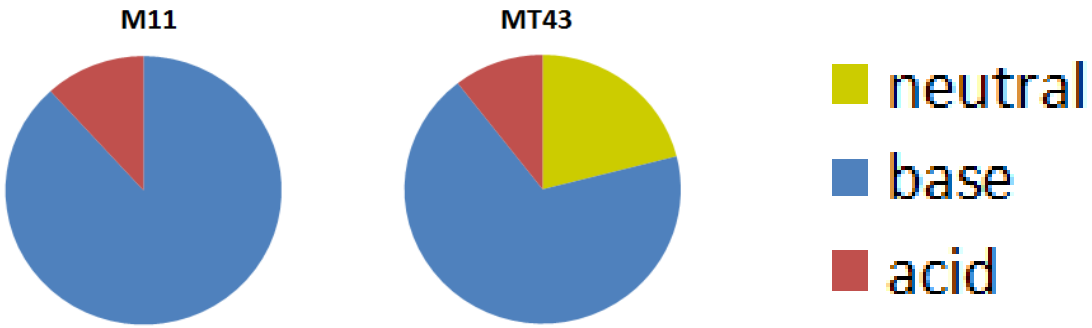
Lacosamide, meloxicam, alprazolam, indomethacin and carbamazepine not metabolized well by HLM or BM3 CYPs

Glipizide , diclofenac, aprepitant, not improved

Metabolism of many compounds improved with BM3.

Ariprazole not different HLMs and bacterial CYPs

Trend Visible in Charges of Drugs Metabolized by BM3 CYPs



Charge distribution of drugs turned over >20%

M11 Ser72 to MT43 Asp72 (polar residue to acidic residue)

There are substrate preferences between mutants

Metabolic Activity and Diversity of 9 Probe Drugs by 14 BM3 Mutants

Compound aiu	M01	MT41 (A74E)	MT43 (S72D)	MT44 (S72E)	M02	M05	M11	MT32 (L437E)	MT33 (L437N)	MT35 (L437S)	MT36 (L437T)	MT34 (A74E)	MT37 (A74D)	MT38 (S72D)	HLM [b]
OND	11	7	37	14	12	1	4	6	12	3	2	8	1	16	
(%)[c]	1		1		1	3	3	3		4	3			1	√
M+16_1	3		3		2	5	5	4		6	4			1	√
M+16_2	10	2			2	7	8	4	10	11	14	2	2	2	√
M+16_3	31	20	6	7	22	20	21	11	20	19	25	13	21	15	√
M+16_4	28	60	50	44	28	18	18	9	12	9	8	22	49	26	√
M+16_5	3			16								15		14	√
M+16_6		5	8	9	12	4		13	8	8	4	14	6	12	√
M+16_7			6	3	1		4							2	N
BUS	56	39	56	33	91	62	25	47	3	33	18	1	19	13	
(%)[c]	2	16	25	26	8	1		4		2	2	5	37	35	√
M+16_1		8	15	14	8										√
M+16_2		35	25	6	65							3			√
M+16_3		16	16	22	10	85		90	94	91	88	54	39	45	√
M+16_4	71	9	14	16	3	10	79	2		3	5	21	24	20	√
M+16_5	16				6		14								√
M+32		16	5	16		4			6	4	5	17			√
	11						7								

High similarities observed for M01 and M11 in the metabolism of odansetron and buspirone

Alteration of Specific Amino Acid Residues Change the Distribution of Metabolites Formed

Compound aiu	M01	MT41 (A74E)	MT43 (S72D)	MT44 (S72E)	M02	M05	M11	MT32 (L437E)	MT33 (L437N)	MT35 (L437S)	MT36 (L437T)	MT34 (A74E)	MT37 (A74D	MT38 (S72D	HL M [b]
BUS	56	39	56	33	91	62	25	47	3	33	18	1	19	13	
(%)[c]	2	16	25	26	8	1		4		2	2	5	37	35	√
M+16_1		8	15	14	8										√
M+16_2		35	25	6	65							3			√
M+16_3	71	16	16	22	10	85	79	90	94	91	88	54	39	45	√
M+16_4	16	9	14	16	3	10	14	2		3	5	21	24	20	√
M+16_5					6										√
M+32	11	16	5	16		4	7		6	4	5	17			√

Mutations at the Leu437and Ser72 positions of M01 and of Ala74 and Ser72 of M11 result in formation of all five aliphatic hydroxylations whereas M01 and M11 produce primarily aromatic oxidations

Partial Correlation Matrix Based on the Drug Library Screen Results

	M01	M02	M11	MT35	MT38	MT43
M01	1	0.5595	**0.7397**	0.4382	0.4081	**0.4394**
M02		1	0.5082	0.2476	0.3664	**0.4829**
M11			1	0.5540	0.3311	**0.2600**
MT35				1	0.3345	**0.1829**
MT38					1	**0.7549**
MT43						1

Compound [a]	M 01	MT41 (A74E)	MT43 (S72D)	MT44 (S72E)	M02	M05	M11	MT32 (L437E)	MT33 (L437N)	MT35 (L437S)	MT36 (L437T)	MT34 (A74E)	MT37 (A74D)	MT38 (S72D)	HLM [b]
OND	1	7	37	14	12	1	4	6	12	3	2	8	1	16	
(%)[c]	1		1		1	3	3	3		4	3			1	√
M+16_1	1		3		2	5	5	4		6	4			1	√
M+16_2	3	2			2	7	8	4	10	11	14	2	2	2	√
M+16_3	1	20	6	7	22	20	21	11	20	19	25	13	21	15	√
M+16_4	0	60	50	44	28	18	18	9	12	9	8	22	49	26	√
M+16_5	3			16								15		14	√
M+16_6	1	5	8	9	12	4	4	13	8	8	4	14	6	12	√
M+16_7	2		6	3	1									2	N
M+32_1	8		2	1											√
M+32_2		4	11	4	10	22	21	25	29	20	22	9	12	5	√
M-14_1	3	2			7	16	13	11		11	13			2	N
M-14_2		6	8	10	15	5	6	18	11	11	6	19	9	13	√
M+38		1	5	6			1	2	10	1	1	6	1	7	N
REP	1	7	2	1	6	8	11	12	11	36	5	5	1	4	
(%)[c]	6	100			8	16	16	10	10	8	10		100		√
M+16_1	2		20		2	1			2	2					√
M+16_2	0		8	25	68	45	42	66	56	72	37	100		46	√
M+16_3	5							4	7	7	38				√
M+16_4	8		72	75	22	38	42	20	25	11	15			54	N

Summary of 43 Drug Screening results

- Many compounds are metabolized more efficiently for the BM3 mutants tested
- For buspirone and ondansetron high similarities were observed for M01 and M11 whereas significant differences were observed for other mutants
- Mutations at the Leu437 and Ser72 positions improved activity towards most drugs tested relative to M11 and improved metabolite diversity
- Mutations at 2 active site positions of M01 and M11 changed the profiles from aromatic to aliphatic oxidations for a few substrates (buspirone most pronounced)
- Significantly altered metabolic profiles for buspirone, ondansetron, popafenone and repaglinide were generated by different mutants
- These results demonstrate that the mutants described have very promising properties for drug library diversification and production of human relevant metabolites of drugs

Acknowlegements

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John Saunders
Lifang Sun
Nico Vermeulen
Jan Commandeur
Sam Sperry

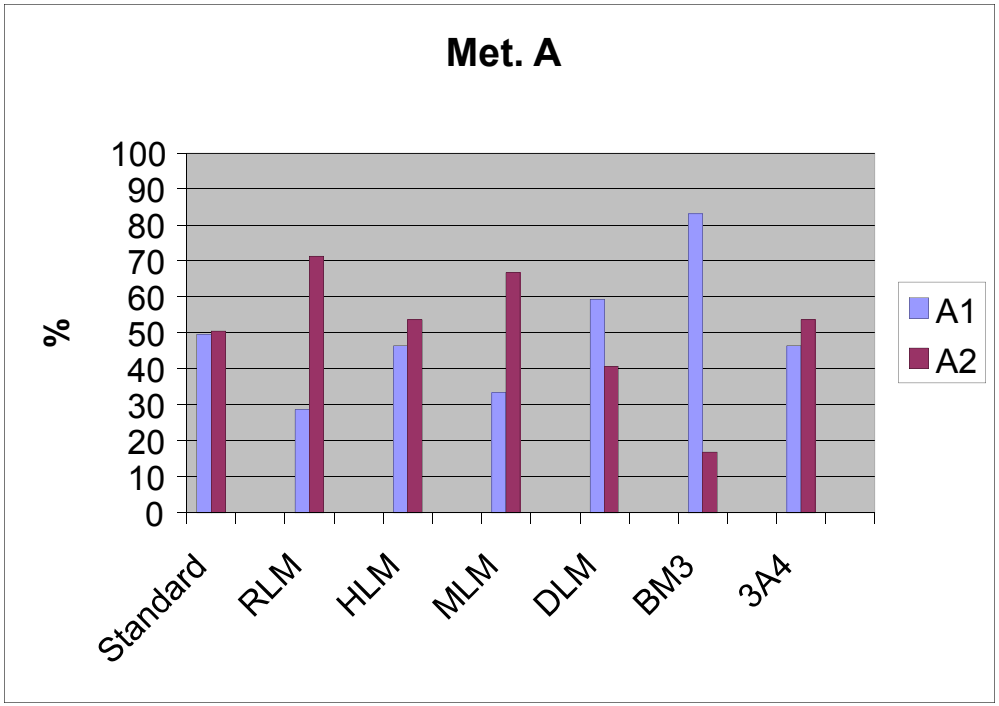
Backups

Mutations

R47L	R47L	R47L	R47L	R47L	R47L	R47L	R47L	R47L	R47L	R47L	R47L	R47L	R47L
			E64G	E64G	E64G	E64G	E64G	E64G	E64G	E64G		S72D	S72E
						A74E			A74D		A74E		
		F81I	F81I	F81I	F81I	F81I	F81I	F81I	F81I	F81I			
	L86I												
F87V	F87V	F87V	F87V	F87V	F87V	F87V	F87V	F87V	F87V	F87V	F87V	F87V	F87V
			E143G	E143G	E143G	E143G	E143G	E143G	E143G	E143G			
L188Q	L188Q	L188Q	L188Q	L188Q	L188Q	L188Q	L188Q	L188Q	L188Q	L188Q	L188Q	L188Q	L188Q
			Y198C	Y198C	Y198C		Y198C	Y198C		Y198C			
E267V		E267V	E267V	E267V	E267V	E267V	E267V	E267V	E267V	E267V	E267V	E267V	E267V
			H285Y	H285Y	H285Y		H285Y	H285Y		H285Y			
	N319T												
G415S		G415S	G415S	G415S	G415S	G415S	G415S	G415S	G415S	G415S	G415S	G415S	G415S
				L437E	L437N		L437S	L437T					
	A964V												
		G1049E											

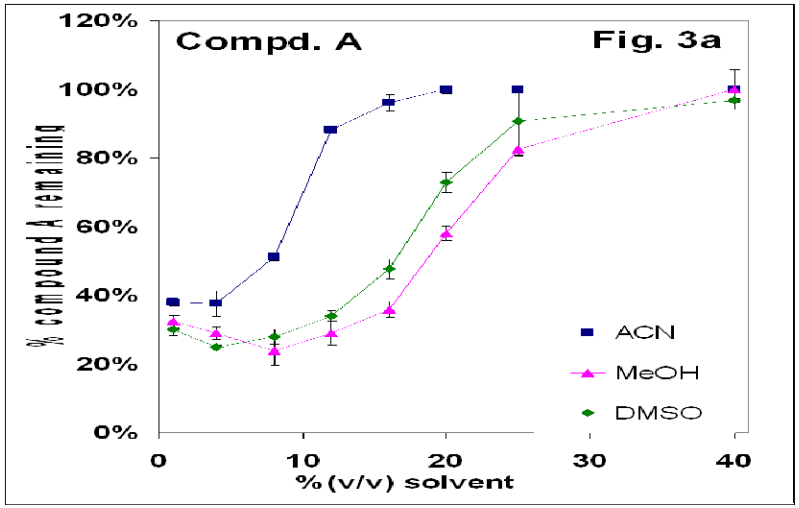
Enantiomer Ratios for for Metabolite A (endo)

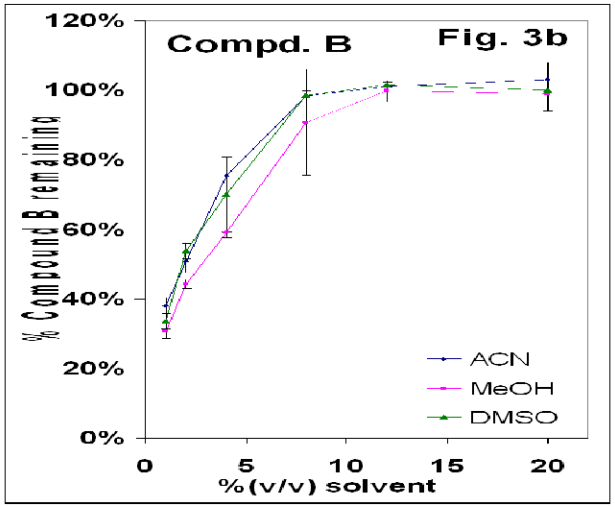
	Peak area ratio(A1/A2)	
	A1	**A2**
Standard	49.61	50.39
RLM	28.67	71.34
HLM	46.34	53.66
MLM	33.37	66.63
DLM	59.28	40.72
BM3	83.14	16.86
3A4	46.42	53.58

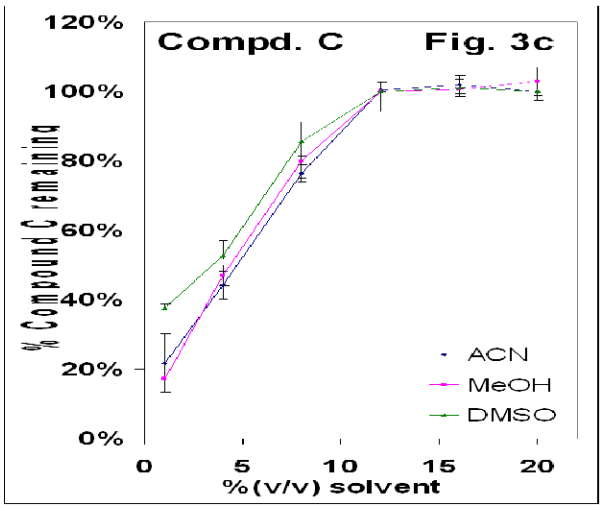


Ratio of A2 to A1 ~ 6 in dogs orally administered 10 mg/kg of compound A

Formation of metabolites of Compound A, Metabolite B or Metabolite C with Different Solvent Concentrations







The conversion of Compound B (endo) and Compound C (exo) by BM3/M11 (i.e secondary metabolism) is more sensitive to the addition of solvent than is the conversion of Compound A

Compound [a]	M01	MT41 (A74E)	MT43 (S72D)	MT44 (S72E)	M02	M05	M11	MT32 (L437E)	MT33 (L437N)	MT35 (L437S)	MT36 (L437T)	MT34 (A74E)	MT37 (A74D)	MT38 (S72D)	HLM [b]
AMI	59	59	48	54	53	89	70	80	65	93	72	81	73	92	
(%)[c]	5	13	13	5	9	23	8	15	10	19	15	14	11	14	√
M+16_1	1	2	2	1	6	2	1	1	2	3	3	1	1	1	N
M+16_2	16	10	13	16	14	9	7	7	5	4	6	10	11	16	√
M+16_3							7		1			1	2		√
M+16_4		10	3	3				1	2		3				N
M+32_1	1	1	1		1	1	1	2		4	1	1	1	1	N
M+32_2	65	53	58	64	57	50	68	60	71	58	63	65	66	55	√
M-14	12	11	10	11	13	15	8	14	9	12	9	8	8	13	N
BUS	56	39	56	33	91	62	25	47	3	33	18	1	19	13	
(%)[c]	2	16	25	26	8	1		4		2	2	5	37	35	√
M+16_1		8	15	14	8										√
M+16_2		35	25	6	65							3			√
M+16_3	71	16	16	22	10	85	79	90	94	91	88	54	39	45	√
M+16_4	16	9	14	16	3	10	14	2		3	5	21	24	20	√
M+16_5					6										√
M+32	11	16	5	16		4	7		6	4	5	17			√
CLZ	7	36	87	75	27	24	5	62	7	34	29	19	4	75	
(%)[c]	69	88	88	91	65	86	85	88	78	86	84	94	100	97	√
M+16_1	6	4	4	4	9	7	7	3	5	5	6	6		3	√
M+16_2	25	8	3	5	26	7	8	9	17	9	10				√
M+16_3			5												N

Compound [a]	M01	MT41 (A74E)	MT43 (S72D)	MT44 (S72E)	M02	M05	M11	MT32 (L437E)	MT33 (L437N)	MT35 (L437S)	MT36 (L437T)	MT34 (A74E)	MT37 (A74D)	MT38 (S72D)	HLM [b]
CTP	2	28	20	24	1	30	7	47	12	46	31	6	11	51	
(%)[c]	100	3	5	4	12	2	2	2	2	2	2	5	4	4	√
M+16		95	93	93	88	95	97	88	94	91	92	95	96	88	√
M-14		2	2	3		3	1	10	**4**	**7**	**6**			**8**	N
M-28															
DEX	44	19	5	35	24	70	39	60	46	74	62	50	22	42	
(%)[c]	1	2	1	1	3			1	1	1	1	1	1	1	N
M+16_1	3	1	11	2	9	1	1	2	5		1	6	2	1	√
M+16_2		5		1		2	2	3		6	5	1	16	10	√
M+16_3	96	86	88	88	88	96	96	90	90	90	91	85	72	78	N
M-14_1									4			1	3	1	√
M-14_2	1	5		7		1	1	2		1	1	5	6	7	N
M+2_1		1		1				2		2	1	1	1	2	N
M+2_2															
DIL	1	13	11	16	1	1	2	24	31	43	4	1	8	19	
(%)[c]	8	2	3	2		9	9	3	2	2	4			4	√
M-14_1	92	96	97	97	100	91	91	93	94	87	96	100	100	92	√
M-14_2								1		1				1	√
M-28_1		2		1				4	4	10				3	√
IRB	16	52	9	21	1	37	15	72	32	69	46	15	15	38	
(%)[c]	99	98	99	99	94	99	97	99	99	99	99	98	95	99	√
M+16	1	2	1	1	6	1	3	1	1	1	1	2	5	1	N

Substrate Depletion Results for Metabolism of 43 Drugs by 6 BM Mutants 2

Compound	M01[a]	M02[a]	M11[a]	MT35[a]	MT38[a]	MT43[a]	HLMs
Minaprine	11	6	<5 [b]	19	7	9	<5
Nelfinavir	7	<5 [b]	<5 [b]	<5 [b]	24	37	<5 [b]
Nicotine	36	8	<5 [b]	43	37	21	48
Nifedipine	63	32	<5 [b]	<5 [b]	58	67	<5 [b]
Nilotinib	9	23	<5 [b]	21	10	10	24
Ondansetron	30	6	11	9	18	30	6
Paroxetine	10	6	41	17	17	6	<5 [b]
Phenacetine	<5 [b]	<5 [b]	<5 [b]	<5 [b]	<5 [b]	6	9
Pimozide	16	38	<5 [b]	26	37	11	<5 [b]
Propafenone	10	<5 [b]	21	23	22	21	<5 [b]
Quinidine	<5 [b]	17	9	23	<5 [b]	<5 [b]	<5 [b]
Repaglinide	<5 [b]	<5 [b]	13	55	<5 [b]	<5 [b]	33
Rosiglitazone	99	99	95	82	76	99	<5 [b]
R-warfarin	6	<5 [b]	<5 [b]	<5 [b]	<5 [b]	<5 [b]	<5 [b]
Saquinavir	<5 [b]	<5 [b]	<5 [b]	35	<5 [b]	8	52
Sorafenib	<5 [c]	<5 [b]	36	<5 [b]	38	36	71
Tamoxifen	62	68	36	28	63	73	8
Terfenadine	10	21	7	7	62	54	<5 [b]
Thioridazine	99	91	96	97	97	83	7
Tipranavir	23	<5 [c]	<5 [b]	63	63	69	58
Verapamil	<5 [b]	<5 [b]	<5 [b]	27	<5 [b]	<5 [b]	30

Method used for Compound A:

Biotransformation: shake to incubate for 22 hr at 24°C.

↓

Protein removal: precipitate with 2.5 Vol of ACN, centrifuge.

↓

Solid-phase extraction: evaporate ACN, extract with 10g C_{18} SPE cartridges, elute with ACN.

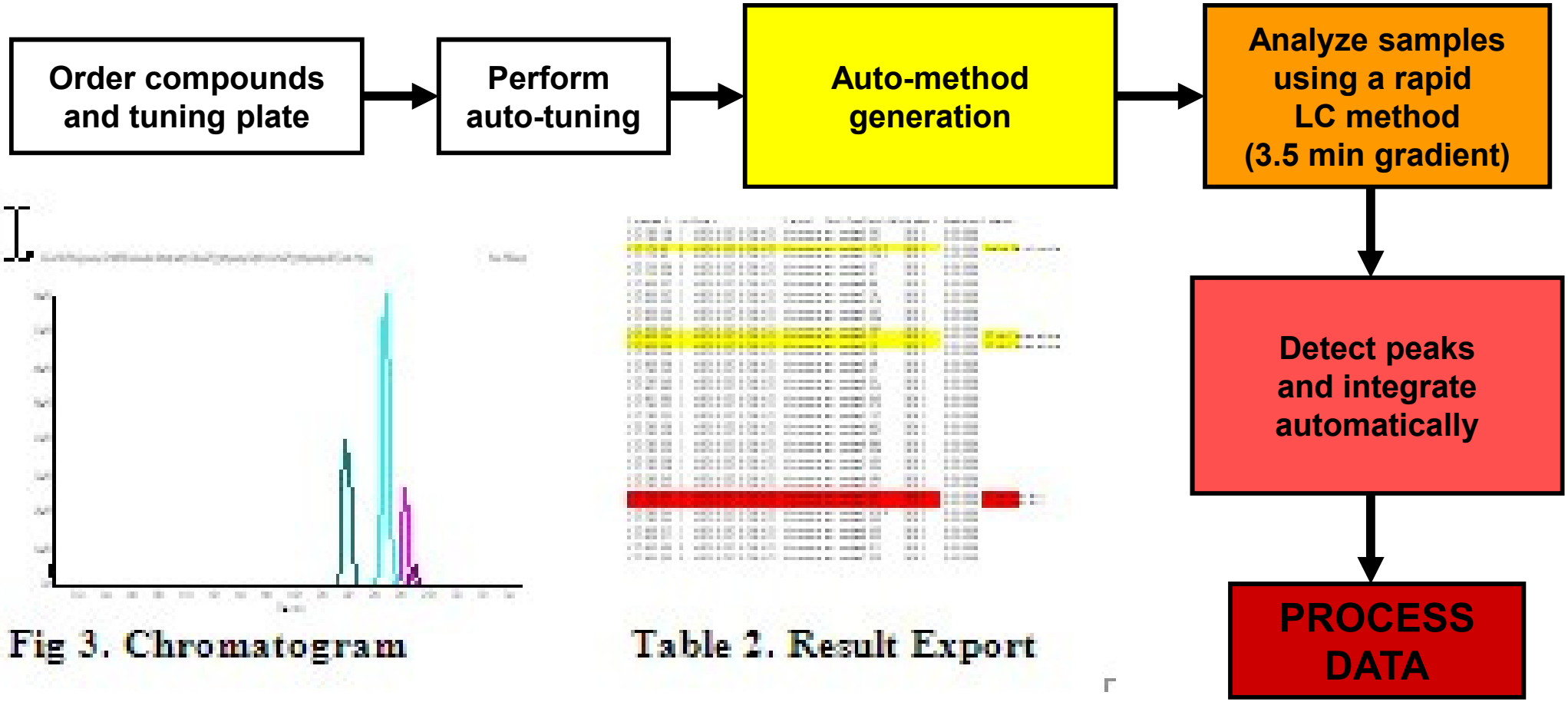
↓

HPLC purification: Evaporate ACN, redissolve solid residue in DMSO, purify by reversed-phase HPLC.

Limitations:

1. Incomplete removal of proteins
2. Cannot reuse enzyme
3. Labor-intensive and difficult to automate

High Throughput LS/MS/MS Screening Used to Monitor Substrate Depletion



Yongmin Li et al,
ASMS, Seattle 2006