Study Report MPF/DDM 0010: In Vitro Metabolism of BSF 208075 in Rat, Dog and Human Microsomes and Hepatocytes

Study Investigator and Site:

[redacted]

Objective

The study was performed to investigate the in vitro metabolism of BSF 208075 in liver microsomes and hepatocytes of rat, dog and humans.

Methods

Human liver microsomes were obtained from [redacted]. They were pooled from livers of female and male donors. The livers were considered intact and able to metabolize normally. The protein content was 20mg/mL and the CYP P450 content was 0.43 nM/mg protein.

Human CYP 450 enzymes and UGT enzymes with defined protein and enzyme contents were obtained from [redacted]. Microsomes from cell lines containing [redacted] 1.

Human hepatocytes were obtained from 3 patients (2 males and 1 female) undergoing partial hepatectomy. They were in the age of 21, 51 and 72 years and 2 of the 3 individuals were smokers.

In the incubations with microsomes the incubation mixture contained the following:

[redacted]

[redacted]

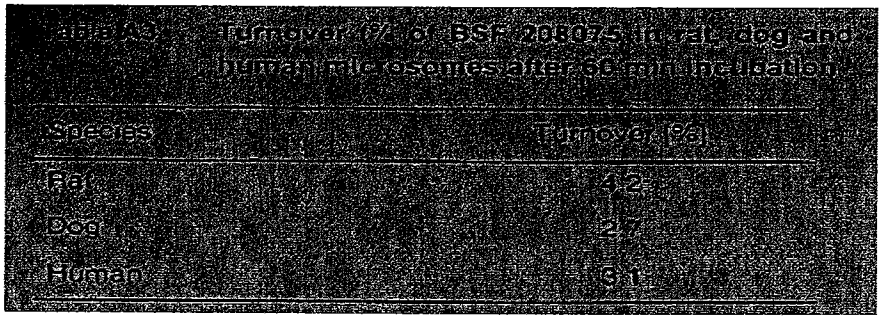
[redacted]

[redacted]

Incubations with microsomes from cells expressing single glucuronyltransferases were performed to investigate whether BSF 208075 is a substrate of Phase II enzymes. Seven different UGT isoenzymes were tested.

Results

The turnover of BSF 208075 in incubations of human microsomes was small (3-4%) as shown in Table A3:



Species	Turnover (%)
Rat	4.2
Dog	2.7
Human	3.1

suggesting that the main metabolic pathway of BSF 208075 is not by Phase I enzymes. CYP 3A4, 3A5 and 2C19 were the identified main Phase I enzymes.

Incubation of BSF 208075 with human hepatocytes resulted in a turnover of 21% in 24 h generating 6 metabolites as shown in Table A6:

Table A6 Turnover (%) of BSF 208075 in rat, dog and human hepatocytes after 4 h, 8 h and 24 h incubation of 10^{-4} and 5×10^{-5} M BSF 208075

Incubation time [h]	Rat (pool)		Dog (pool)		Human					
					Donor 1 (male)		Donor 2 (female)		Donor 3 (male)	
	5×10^{-5} M	10^{-4} M	5×10^{-5} M	10^{-4} M	5×10^{-5} M	10^{-4} M	5×10^{-5} M	10^{-4} M	5×10^{-5} M	10^{-4} M
4	3.3	4.1	6.8	6.0	–	–	6.9	3.2	3.8	3.4
8	8.4	7.8	14.8	10.3	5.4	6.2	10.3	5.8	7.2	9.9
24	17.5	12.0	27.9	21.9	17.9	18.8	28.9	14.7	21.2	27.2
8	11.4	8	24.9	–	–	–	12.1	–	–	–

– no 4-h samples available

One of the metabolites (H2) was identified as a glucuronide of BSF 208075 and 2 other metabolites (H3 and H4) were regioisomers of the glucuronide and the remainder metabolites were only present in small amounts as shown in Table A7:

Table A7 Metabolites (% of the radioactivity in the incubation supernatants) in rat, dog and human hepatocytes after 24 h incubations (pool of 3 individuals/species; mean of two doses)

Species	H1	H2	H3	H4	H5	H6	BSF 208075 + H7
Rat	0.4	1.8	1.3	3.0	0.8	7.3	85.3
Dog	1.1	7.8	3.7	9.1	1.5	–	75.1
Human	0.3	7.5	3.2	8.4	1.4	0.7	80.1

The structure of the suspected main metabolites is shown in the below scheme:

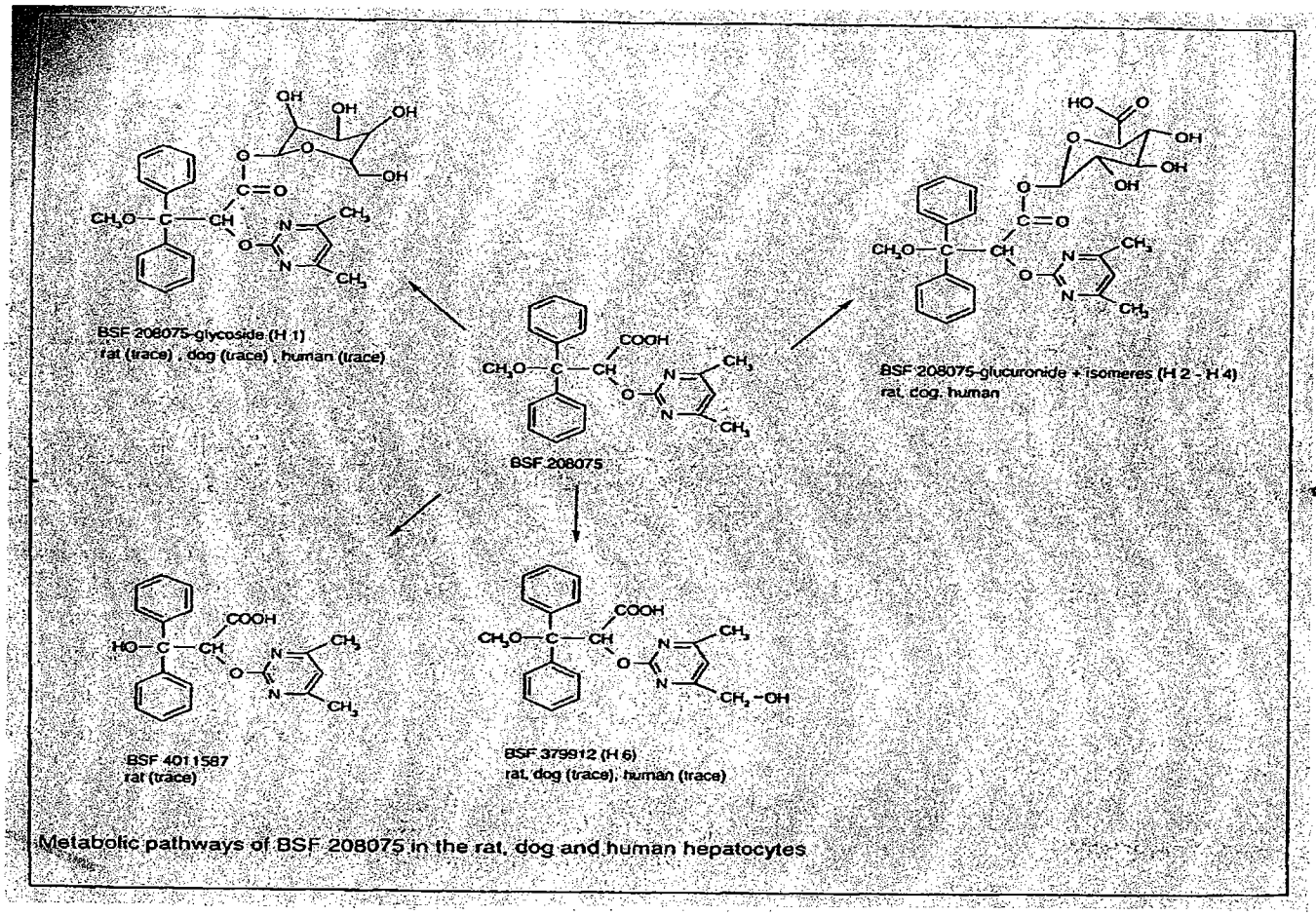


Table A8 lists the turnover rates of BSF 208075 by the individual glucuronyltransferases:

Table A5 Turnover (%) and turnover rate [nmol/min/mg protein] of BSF 208075 in GENTEST microsomes containing single human glucuronyltransferases

UGT isoenzymes	Turnover [%]	Turnover rate [nmol/min/mg protein]
UGT1A1	–	–
UGT1A15	–	–
UGT1A3S	4.2	0.02
UGT1A4	2.7	0.01
UGT1A6	–	–
UGT1A6S	–	–
UGT1A9	7.2	0.01
UGT1A9S	17.8	0.07
UGT2B7S	6.6	0.03
UGT2B15S	–	–

– not detectable

The results indicated that UGT1A9S, UGT1A9 and UGT2B7S are the most important Phase II enzymes involved.

Conclusion

The extent of CYP mediated Phase I metabolism of ambrisentan is small. CYP 3A and 2C19 are involved. Metabolism of ambrisentan by Phase II metabolism involving glucuronyltransferases is more important.

Comments

None

Study Report: Inhibition of cDNA-Expressed Cytochroms P4501A2, P4502A6, P4502B6, P4502C8, P4502C9, P450 2C19, P4502D6, P4502E1, P4503A4 and CDNA Expressed Uridine Glucuronosyl Transferases 1A1, 1A6, 1A9 and 2B7 Catalaytic Activities by the Test Substance BSF 208075

Study Investigator and Site:

[redacted]

Objectives

To determine whether BSF 208075 inhibits CYPP 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, UGT1A1, UTG 1A6, UGT1A9 and UGT2B7

Methods

The inhibition of the enzymes was tested using model substrates and cDNA derived enzymes in microsomes prepared from human lymphoblastoid cell lines or baculovirus-infected insect cells. The degree of inhibition of the enzymes was tested at 9 concentrations of BSF 208075 0, 0.1, 0.3, 1, 3, 10, 30, 100 and 300 µM (range 0-113.4 µg/mL).
A single concentration of the model substrate (near the Km value) was tested in duplicate. A comparator (known inhibitor or substrate) was tested in duplicate. Metabolism of the model substrates were assayed by measuring the production of a metabolite via [redacted] via HPLC separation with [redacted] spectrometry. Negative controls or blanks, as well as duplicate samples provided control for bias.

Enzymes and substrates used are shown in the below table:

Enzyme	Substrate	[redacted]
CYP1A2	Phenacetin	M103c
CYP2A6	Coumarin	P254
CYP2B6	(S)-Mephenytoin	P255
CYP2C8	Paclitaxel	P212
CYP2C9	Diclofenac	M118r
CYP2C19	(S)-Mephenytoin	P259
CYP2D6	Bufuralol	M117r
CYP2E1	p-Nitrophenol	P206
CYP3A4	Testosterone	M107r
UGT1A1	7-hydroxy trifluoromethyl coumarin	P411
UGT1A6	7-hydroxy trifluoromethyl coumarin	M316a
UGT1A9	7-hydroxy trifluoromethyl coumarin	M319a
UGT2B7	7-hydroxy trifluoromethyl coumarin	P427
Control Microsomes	Added to standardize protein concentration	M101b

The positive controls used are listed in the below table:

Enzyme	Positive Control	Concentrations
CYP1A2	7,8-Benzoflavone	0.3 µM
CYP2A6	Tranylcypromine	100 µM
CYP2B6	Tranylcypromine	100 µM
CYP2C8	Quercetin	30 µM
CYP2C9	Sulfaphenazole	3 µM
CYP2C19	Tranylcypromine	100 µM
CYP2D6	Quinidine	1 µM
CYP2E1	4-Methylpyrazole	50 µM
CYP3A4	Ketoconazole	1 µM
UGT1A1	Bilirubin	50 µM
UGT1A6	Naphthol	50 µM
UGT1A9	Propofol	50 µM
UGT2B7	Eugenol	100 µM

Positive controls for inhibition of each enzyme were used. Percent inhibition was calculated for each replicate test sample. All comparisons were made with the mean of the 0 µM samples representing 100% enzyme activity.

Results

The results on the inhibition of each of the tested enzymes by BSF 208075 are shown In Tables 1-13 (first column: concentration of BSF 208075, second column= concentration of substrate):

Table 1. Inhibition of cDNA-expressed CYP1A2

Concentration (μM)	Pmoles / Incubation	Percent Inhibition
0	494, 466	
0.1	490, 477	2.1, 0.6
0.3	500, 580	-4.2, -21
1	497, 518	-3.5, -7.9
3	499, 518	-4.0, -7.9
10	628, 512	-31, -6.7
30	483, 504	0.6, -5.0
100	594, 554	-24, -15
300	482, 441	0.4, 8.1
Positive Control 7,8 Benzoflavone		
0	476, 532	
0.3	13, 12	97, 98

Table 2. Inhibition of cDNA-expressed CYP2A6

Concentration (μM)	Pmoles / Incubation	Percent Inhibition
0	30.9, 29.8	
0.1	30.6, 29.5	0.7, 3.0
0.3	28.7, 29.2	5.6, 3.9
1	29.7, 28.0	2.3, 7.9
3	30.7, 31.1	-1.0, -2.3
10	31.8, 35.5	-4.6, -17
30	30.1, 33.4	-1.0, -9.9
100	27.2, 29.5	11, 3.0
300	22.9, 26.3	25, 13
Positive Control Tranylcypromine		
0	20, 24	
100	0.2, 0	99, 100

Table 3. Inhibition of cDNA-expressed CYP2B6

Concentration (μM)	Pmoles / Incubation	Percent Inhibition
0	85, 108	--
0.1	104, 126	-7.2, -30
0.3	110, 83	-13, 14
1	108, 127	-11, -31
3	102, 89	-5.2, 8.2
10	92, 104	5.2, -7.2
30	126, 110	-30, -13
100	105, 113	-8.2, -16
300	95, 159	2.1, -64
Positive Control-Tranylcypromine		
0	80, 149	--
100	5.5, 0	95, 100

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Table 5. Inhibition of cDNA-expressed CYP2C8

Concentration (μM)	Pmoles / Incubation	Percent Inhibition
0	111, 113	--
0.1	112, 116	0, -3.6
0.3	112, 111	0, 0.9
1	116, 124	-3.6, -11
3	123, 117	-10, -4.6
10	115, 110	-2.7, 1.8
30	111, 117	0.9, -4.5
100	118, 113	-5.4, -0.9
300	96, 100	14, 11
Positive Control-Sulfaphenazole		
0	122, 133	--
3	8.4, 11	93, 91

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Table 6. Inhibition of cDNA-expressed CYP2C19

Concentration (μM)	Pmoles / Incubation	Percent Inhibition
0	1263, 1300	—
0.1	1292, 1263	0.8, 1.5
0.3	1248, 1317	2.7, 2.7
1	1397, 1280	-9.0, 0.2
3*	1330, 1183	-3.7, 7.7
10	1324, 1240	-3.3, 5.3
30	1325, 1322	-3.4, -3.1
100	1207, 1279	5.9, 0.2
300	1292, 1290	-0.8, -0.6
Positive Control - Tranylcypromine		
0	1286, 1434	—
100	76, 96	94, 93

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Table 7. Inhibition of cDNA-expressed CYP2D6

Concentration (μM)	Pmoles / Incubation	Percent Inhibition
0	280, 269	
0.1	269, 272	2.2, -1.1
0.3	279, 282	0.4, -2.5
1	265, 274	3.6, -0.4
3*	274, 253	0.7, 5.9
10	262, 284	5.5, -3.8
30	276, 285	0.4, -4.0
100	284, 280	-3.3, -2.6
300	274, 284	0.4, -3.3
Positive Control - Quinidine		
0	237, 239	
1	17, 18	93, 92

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Table 8. Inhibition of cDNA-expressed CYP2E1

Concentration (µM)	Pmoles / Incubation	Percent Inhibition
0	5016, 5244	
0.1	5244, 5168	2.2, -0.7
0.3	5168, 5016	0.7, 2.2
1	5168, 5396	0.7, -5.2
3	5244, 5472	2.2, -6.7
10	5244, 5396	2.2, -5.2
30	5624, 5396	-9.6, -5.2
100	5472, 5472	-6.7, -6.7
300	5168, 5320	0.7, -3.7
Positive Control – 4-methylpyrazole		
0	3040, 2964	
50	380, 380	87, 87

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Table 9. Inhibition of cDNA-expressed CYP3A4

Concentration (µM)	Pmoles / Incubation	Percent Inhibition
0	824, 807	
0.1	761, 757	6.7, 7.2
0.3	714, 714	[illegible]
1	720, 706	12, 14
3	786, 804	[illegible]
10	738, 734	9.6, 10
30	724, 822	[illegible]
100	[illegible], 745	[illegible]
300	865, 773	[illegible]
Positive Control – ketoconazole		
0	983, 1341	
1	188, 182	84, 84

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Table 10. Inhibition of cDNA-expressed UGT1A1

Concentration (μM)	Pmoles / Incubation	Percent Inhibition
0	1247, 1267	--
0.1	1320, 1221	5.9, 2.9
0.3	1286, 1262	2.3, 0.4
1	1257, 1234	0.1, 0
3	1356, 1226	7.9, 2.5
10	1251, 1278	0.4, 1.7
30	1137, 1220	9.5, 2.9
100	1085, 1037	14, 13
300	898, 870	29, 31
Positive Control – Bilirubin		
0	1061, 1074	--
50	82, 52	92, 95

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Table 11. Inhibition of cDNA-expressed UGT1A6

Concentration (μM)	Pmoles / Incubation	Percent Inhibition
0	1854, 1980	--
0.1	1851, 1941	3.4, 1.2
0.3	1977, 1985	-3.1, -3.6
1	1852, 1825	3.4, 4.8
3	1891, 1850	1.9, 3.5
10	1777, 1920	7.3, 0.2
30	1864, 1731	2.8, 10
100	1906, 1743	0.6, 9.1
300	1697, 1725	11, 10
Positive Control – 1-Naphthol		
0	1380, 1521	--
50	68, 69	95, 95

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Table 12. Inhibition of cDNA-expressed UGT1A9

Concentration (µM)	Pmoles / Incubation	Percent Inhibition
0	855, 847	
0.1	836, 893	1.7, 4.9
0.3	857, 890	-0.6, -4.5
1	883, 873	-3.8, -2.6
3	854, 880	0.3, -3.4
10	878, 875	-3.2, -2.8
30	839, 846	1.0, 1.0
100	769, 767	10, 10
300	654, 646	23, 24
Positive Control - Propofol		
0	849, 900	
50	536, 537	39, 39

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Table 13. Inhibition of cDNA-expressed UGT2B7

Concentration (µM)	Pmoles / Incubation	Percent Inhibition
0	3214, 3014	
0.1	3171, 2967	1.6, 4.7
0.3	2949, 3164	5.3, -1.6
1	3266, 3096	-4.9, 0.6
3	3311, 3232	-6.3, -3.8
10	2999, 3052	3.7, 2.0
30	2794, 3251	10, -4.4
100	2540, 2993	18, 3.9
300	2813, *	9.7,
Positive Control - Eugenol		
0	3409, 3556	
100	1886, 1929	46, 45

* Duplicate Sample lost

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Of the tested Phase I enzymes only CYP2A6 and 2C9 were inhibited by about 10-20 % at the highest concentrations of BSF 208075 (300 μM). UGT1A1 was inhibited up to 30 % at the highest concentration of BSF 208075. UGT1A6, 1A9 and 2B7 were inhibited by about 10-20% at the highest concentrations of BSF 208075. The projected IC_{50} for the inhibition of all tested enzymes exceeds 300 μM (113.4 μg/mL)

Conclusion

The mean steady-state Cmax of ambrisentan in healthy volunteers after administration of 10 mg qd is about 700 ng/mL. Thus, the in vivo inhibition of the tested Phase I and II Phase enzymes by ambrisentan is not projected to be strong. However, it should be noted that ambrisentan was not preincubated in the experiments.

Comments

1. BSF 20875 was not pre-incubated.

2. None of the substrates used is on the list of recommended substrates (Guidance for Industry, Drug Interaction Studies- Study Design, Data Analysis, and Implications for Dosing and Labeling, September 2006. However, this is not an issue because the studies were perfomed prior to issuance of the Guidance.

3. The inhibitors used for CYP 1A2, 2B6, 2C8, 2C9, and 2E1 did not correspond to those recommended (Guidance for Industry, Drug Interaction Studies- Study Design, Data Analysis, and Implications for Dosing and Labeling, September 2006. However, this is not an issue because the studies were performed prior to issuance of the Guidance.

Study Report: MG-1002: The Effect of Ambrisentan, Darusentan, Bosentan, and Sitaxsentan on the Hepatobiliary Disposition of Probe Substrates in Sandwich Cultured Human Hepatocytes

Study Investigator and Study Site: [redacted]

Sponsor: Myogen, Research and Development
Westminster, CO

Objective

To compare the effects of ambrisentan, darusentan, bosentan and sitaxsentan on the hepatobiliary transport system

Methods

[redacted] technology for the analysis of hepatobiliary disposition was used to evaluate the effects of the endothelin receptor antagonists ambrisentan, bosentan, darusentan and sitaxsentan on the transporter mediated uptake and/ or efflux of various probe substrates.

[redacted]

[redacted]

[redacted]

Results

The impact of ambrisentan, bosentan, darusentan and sitaxsentan on hepatic uptake of taurocholate and estradiol-17β-D-glucuronide and biliary excretion of taurocholate is shown in Tables 1-3:

Table 1. The Effect of Ambrisentan, Darusentan, Bosentan and Sitaxsentan on the Na-dependent Uptake of Taurocholate in Human Hepatocytes (Mean ± Standard Deviation, n=3)

	Dose (μM)	Accumulation in Cell Lysate with sodium (pmol/mg)	Accumulation in Cell Lysate without sodium (pmol/mg)	Difference	SD	% Control	SD
Control	0	231	9.3	221	51.0	100.0	0.0
Positive Control (CycA)	100	26.5	10.4	16.1	21.2	6.6 **	8.4
Ambrisentan	2	230	11.0	219	94.4	96.9	24.1
	20	247	5.90	241	99.5	106	20.1
	100	203	13.3	254	127	109.2	5.89
Darusentan	2	232	15.0	252	96.3	107.2	18.7
	20	267	8.82	258	98.9	115	18.9
	100	242	7.46	235	46.7	107	9.62
Bosentan	2	265	9.18	256	60.4	116	4.73
	20	195	5.08	206	55.4	84.4	3.47
	100	74.3	2.61	79.8	19.0	33.1**	1.96
Sitaxsentan	2	197	9.39	188	33.2	85.6	5.49
	20	51.6	6.98	44.6	25.2	19.4 **	6.89
	100	9.27	4.25	5.02	4.26	2.10 **	1.36

** = p < 0.01

Table 2. The Effect of Ambrisentan, Darusentan, Bosentan and Sitaxsentan on the OATP Mediated Uptake (Intracellular Accumulation) of E2-17βG in Human Hepatocytes (Mean ± Standard Deviation, n=3)

	Dose (μM)	Accumulation in Cell Lysate with Calcium (pmol/mg)	Accumulation in Cell Lysate without Calcium (pmol/mg)	% Control	SD	BEI (%)
Control	0	25	23.7	100.00	0.00	5.41
Ambrisentan	2		21.2	105.0	11.9	
	20		23.4	91.7	18.9	
	100		20.0	85.1	31.6	
Darusentan	2		19.9	97.9	15.6	
	20		22.0	106.1	35.0	
	100		19.6	82.0	17.2	
Bosentan	2		19.6	93.3	127.7	
	20		18.5	71.8	19.2	
	100		21.0	81.5	52.4	
Sitaxsentan	2		17.2	76.0	18.4	
	20		17.1	63.4	28.5	
	100		15.42	52.1	10.27	

* = p < 0.05

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Table 3 The Effect of Ambrisentan, Darusentan, Bosentan and Sitaxsentan on the BSEP Mediated Biliary Excretion of Taurocholate in Human Hepatocytes (Mean ± Standard Deviation, n=3).

	Dose (μM)	Accumulation in Cell Lysate With Calcium (pmol/mg)	Accumulation in Cell Lysate Without Calcium (pmol/mg)	BEI (%)	SD	% Control	SD
Control	0	229	367	40.8	11.4	100.00	0.0
Positive Control (Ritonavir)	100	41.5	51.7	17.2	1.4	50.2	1.5
Ambrisentan	2	59.9	101	47.1	7.21	118.1	14.9
	20	61.9	116	43.2	15.7	105.2	21.8
	100	94.3	164	44.4	6.15	112	17.4
Darusentan	2	117	221	47.4	7.41	119	13.4
	20	134	220	40.3	12.8	98.6	15.6
	100	127	221	43.5	4.73	110	16.5
Bosentan	2	82.4	166	48.4	9.31	120	10.6
	20	99.9	168	42.4	10.70	105.1	11.7
	100	47.4	67.9	31.8	8.40	78.11	4.51
Sitaxsentan	2	104	207	51.9	7.37	131	25.7
	20	25.8	51.9	53.7	7.88	135	21.6
	100	5.72	9.10	30.3	21.27	85.3	64.5

* = $p < 0.05$

Sitaxsentan (20 μM and 100 μM) and bosentan (100 μM) significantly inhibit the NTCP mediated hepatic uptake of taurocholate in human hepatocytes. The > 90% inhibition by sitaxsentan is comparable to that by cyclosporine A. No overt inhibition of the NTCP mediated transport of taurocholate is exerted by ambrisentan and darusentan. Among the 4 endothelin receptor blockers only sitaxsentan inhibits the OATP mediated hepatic uptake of estradiol-17β-D-glucuronide importantly by about 50% at the highest concentration of 100 μM. Bosentan at 100 μM inhibits the BSEP mediated biliary excretion of taurocholate significantlyby about 20%. The results obtained for sitaxsentan show large variability. Ambrisentan and darusentan appear not to impact importantly NTCP, OATP or BSEP.

Conclusion

Ambrisentan and darusentan in concentrations up to 100 μM appear not to inhibit NTCP, OATP or BSEP in human hepatocytes importantly. In contrast, sitaxsentan inhibits NTCP and the OATPs significantly. Bosentan can inhibit NTCP and BSEP significantly in human hepatocytes. Sitaxsentan exerted the quantitatively greatest inhibitory effect on NTCP.

Comments

1. No standards for the performance of the sandwich culutured human hepatocytes are provided. It is unclear how the viability of the cells and the functionality and expression of transporters and enzymes of the preparation were controlled. No data acceptance criteria were defined either.

2. There was no positive control included in the experiments investigating the inhibition of the OATP mediated uptake of estradiol-17β-D-glucuronide by the endothelin receptor blockers.

3. The inhibition of MRP2 by the endothelin receptor antagonists was not investigated

4. The statement on p.11 of the report "At the highest concentration studied, sitaxsentan inhibited the transport of probe substrates for NTCP, OATP and BSEP in human hepatocytes" should differentiate between statistically significant and statistically not significant differences.

5. The report should have provided a glossary to explain all abbreviations used.

DPDPE Addendum to Study Report: MG 1002: The Effect of Ambrisentan, Darusentan, Bosentan, and Sitaxsentan on the Hepatobiliary Disposition of Probe Substrates in Sandwich-Cultured Human Hepatocytes

Study Investigator and Site: [redacted]

The MRP family of transport proteins (MRP1-MRP6) is highly regulated during cholestasis. Mrp2 mediates canalicular biliary excretion of organic anions, mostly divalent amphipathic conjugates of glutathione, glucuronate and sulfates. MRP2 may transport cancer chemotherapeutics, uricosurics, antibitotics, and leukotrienes.

Objective

The goal of the study was to determine the effect of ambrisentan, darusentan, bosentan and sitaxsentan on the uptake and efflux of DPDPE, a probe substrate for MRP2.

Methods

[redacted]

1 Page(s) Withheld

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_____ Deliberative Process

Withheld Track Number: Clin Pharm/Bio-_____