

CYP450 inhibition assay: The objective of this study was to determine the potential of FC-12738 to inhibit CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A (with midazolam and testosterone as probe substrate, respectively) in human liver microsomes, using individual probe substrate for each isozyme.

Pooled human liver microsomes were incubated in the presence of FC-12738 (at 0, 0.0300, 0.100, 0.300, 1.00, 3.00, 10.0, 30.0 and 100 μ M), NADPH and a selective substrate for each CYP450 isoform. The formation of the selective metabolite from its substrate was measured by liquid chromatography-tandem mass spectrometry.

CYP time-dependent inhibition assay: The objective of this study was to determine the time-dependent inhibitory effects of FC-12738 on the activities of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A in human liver microsomes using a non-dilution method. Pooled human liver microsomes were pre-incubated for 30 minutes with FC-12738 in the presence or absence of NADPH followed by measurement of remaining enzyme activities.

CYP induction assay: The objective of this study was to evaluate the potential of FC-12738 to induce the mRNAs and activities of CYP450 (CYP) isoforms CYP1A2, CYP2B6, and CYP3A4 in human hepatocytes following in vitro administration. Cryopreserved human hepatocytes prepared from three donors were treated with FC-12738 at 0.100, 1.00, 3.00, 10.0 and 15.0 μ M at 37°C for a total of 48 hours with the change of incubation medium every 24 hours. The incubation solution after every 24-hour was aspirated for the measurement of Lactate Dehydrogenase (LDH) activity. An aliquot of the incubation solutions from the second dosing was taken at 0, 5 and 24 hours after incubating with the hepatocytes for the measurement of concentration change of FC-12738.

After 48 hours incubation, the cells were washed with Hank's balanced salt solution (HBSS) and incubated with a selective substrate for each CYP450 isoform at 37°C for 30 minutes. The formation of a specific metabolite from its substrate in the incubation solution was quantified by LC-MS/MS method. Cells in the cell culture plate were used to evaluate gene expression by real time Polymerase Chain Reaction (PCR).

Assessment of FC-12738 on the activity of P-gp in the Caco-2 cells: Caco-2 cells at passage number 47 were seeded on 96-well transport inserts and cultured for 23 days before being used for transport experiment. After a 120-minute transport experiment, the bi-directional permeability and efflux ratios of digoxin (10.0 μ M), a known P-gp substrate, in the presence of FC-12738 at eight concentrations (0.0457, 0.137, 0.412, 1.23, 3.70, 11.1, 33.3 and 100 μ M) or in the

absence of FC-12738 were determined and LC-MS/MS was used for bioanalysis. The P-gp activity was represented by the efflux ratios of digoxin in the presence and absence of FC-12738 and the %P-gp Activity values were used to calculate IC_{50} .

Assessment of FC-12738 on the activity of BCRP in the Caco-2 cells: Caco-2 cells at passage number 47 were seeded on 96-well transport inserts and cultured for 22 days before being used for the transport experiment. After a 120-min transport experiment, the bi-directional permeability and efflux ratios of estrone 3-sulfate (5.00 μ M), a known BCRP substrate, in the presence of FC-12738 at eight concentrations (0.0457, 0.137, 0.412, 1.23, 3.70, 11.1, 33.3 and 100 μ M) or in the absence of FC-12738 were determined and LC-MS/MS was used for bioanalysis. The BCRP activity was represented by the efflux ratios of estrone 3 sulfate in the presence and absence of FC-12738 and the %BCRP Activity values were used to calculate IC_{50} .

Assessment of FC-12738 on the activity of OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1 and MATE2-K transporters: HEK cells that stably express each transporter (HEK293-OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1, and MATE2-K cells) were incubated with the relevant substrates in the absence and presence of FC-12738. The dosing concentrations of FC-12738 were 0.0457, 0.137, 0.412, 1.23, 3.70, 11.1, 33.3, and 100 μ M. After incubation, concentrations of the substrate in samples were analyzed by LC MS/MS. The %VC (%Vehicle Control, the percentage of the mean transport activity values in the presence and absence of test compound) values were used for half maximal inhibitory concentration (IC_{50}) calculation.

Comparison of TP5 and FC-12738 in plasma and brain in male CD-1 mice after a single IP dose: Three animals were dosed via injection in the lower right quadrant of the abdomen for intraperitoneal injections at 10 mg/kg (10 mL/kg). The peptides were suspended in solutions consisting of 10% N,N-Dimethylacetamide (DMA), 20% propylene glycol (PG), 70% H₂O. All blood samples (30-50 μ L per sample) were taken via appropriate vein (saphenous or submandibular vein) at 0.5, 1, 2, 4, and 8 h for the brain tissue distribution group. Blood samples were collected in Greiner MiniCollect K2EDTA tubes, placed on ice, and within 30 minutes, centrifuged at 15,000g for 5 min to obtain plasma samples. All plasma samples were stored at -70C until analysis. The whole brain was harvested at selected time points, immediately rinsed in water briefly, and blotted dry with a paper towel. The whole brain was weighed and three volumes of PBS buffer (pH 7.4) was added to one volume of each tissue sample which was homogenized by a tissue homogenizer until fine tissue particles were completely dispersed or emulsified. The tissue homogenate samples were stored at -70°C until analysis.

Plasma samples were prepared as follows. Three volumes of acetonitrile containing internal standard was added to one volume of plasma to precipitate proteins. Samples were centrifuged (3000 xg for 10 min) and supernatant was removed for analysis by LCMS/MS. Calibration standards and quality controls were made by preparation of a 1 mg/mL stock solution and subsequently a series of working solutions in methanol : water (1/1, v/v) which were spiked into blank plasma to yield a series of calibration standard samples in the range of 1 ng/mL to 10 µg/mL and quality control samples at three concentration levels (low, middle and high). All incurred PK/PD plasma samples were treated identically to the calibration standards and quality control samples. LC-MS/MS analysis was performed utilizing multiple reaction monitoring for detection of characteristic ions for each drug candidate, additional related analytes and internal standard.

Tissue samples were prepared as follows. Three volumes of PBS buffer (pH 7.4) was added to one volume of each tissue sample which was then homogenized to obtain each tissue homogenate sample.

Subsequently, three volumes of acetonitrile containing internal standard was added to one volume of each tissue homogenate, and the mixture was vortexed, centrifuged (3000 g for 10 min) and supernatant was removed for analysis by LC-MS/MS. Calibration standards were made by preparation of a 1 mg/mL stock solution and subsequently a series of working solutions in methanol : water (1/1, v/v) which were spiked into blank tissue homogenate to yield a series of calibration standard samples in the range of 1 ng/mL to 10 µg/mL. All incurred PK/PD tissue samples were treated identically to the calibration standards. LC-MS/MS analysis was performed utilizing multiple reaction monitoring for detection of characteristic ions for each drug candidate, additional related analytes and internal standard.

Results and Discussion

Caco cell permeability

Caco cells were exposed to increasing concentrations of FC-12738 in the presence or absence of GF120918, a potent

inhibitor of efflux transporter(s), such as P-gp and BCRP. In the absence of GF120918, at 2.00, 10.0 and 100 µM dosing concentrations, FC-12738 showed the mean apparent permeability coefficient (P_{app}) values of

<0.241, 0.107 and 0.0949 × 10⁻⁶ cm/s, respectively, in the apical to basolateral (A to B) direction, and <0.217, 0.0946 and 0.117 × 10⁻⁶ cm/s, respectively, in the B to A direction. Therefore, the efflux ratio (ER_a) couldn't be calculated at the dosing concentration of 2.00 µM. At the dosing concentrations of 10.0 and 100 µM, no efflux was observed as evidenced by the ER_a values of 0.880 and 1.24, respectively. In the presence of GF120918, FC- 12738 showed the mean P_{app} values of <0.237, 0.0900 and 0.0752 × 10⁻⁶ cm/s, respectively, at the three tested dosing concentrations, in the A to B direction, and <0.213, 0.101 and 0.0927 × 10⁻⁶ cm/s, respectively, in the B to A direction. Therefore, the efflux ratio (ER_i) couldn't be calculated at the dosing concentration of 2.00 µM, and the ER_i values were 1.13 and 1.23, respectively, at the dosing concentrations of 10.0 and 100 µM (Table 1). In conclusion, under the conditions tested, FC-12738 demonstrated low permeable across the Caco-2 cell monolayer and was a poor or non-substrate for efflux transporters.

Pharmacokinetics

After IV administration of FC-12738 at 2 mg/kg in male SD rats, FC-12738 showed a plasma clearance (Cl) of 11.8±0.569 mL/min/kg, half-life (T_{1/2}) at 0.318±0.0105 h. The volume of distribution (V_{dss}) was 0.309±0.0165 L/kg, the area under the plasma concentration time curve from time zero to the last quantifiable concentration (AUC_{0-last}) value was 2800±131 ng·h/mL. Following SC administration of FC-12738 at 10 mg/kg in male SD rats, AUC_{0-last} value of FC-12738 was 13000±379 ng·h/mL. The C_{max} value of FC-12738 was 8430±637 ng/mL, while C_{max} was reached at 0.417±0.144 h. The bioavailability was 91.9% at 10 mg/kg. All animals had tolerated the FC-12738 at the dosing levels during the entire course of the study. No adverse effect was observed during the in-life phase of the study. The mean PK parameters of FC-12738 in male SD rats are shown in Table 2.

Table 1: Summary of the bidirectional permeability of FC-12738 in the Caco-2 cells

Conc.(µM)	+ / GF120918	Mean P _{app} (×10 ⁶ cm/s)		Efflux Ratio	Mean %Solution Recovery	
		A to B	B to A		A to B	B to A
2	-	<0.241	<0.217	NA	<94.1	<99.0
	+	<0.237	<0.213	NA	<92.9	<96.5
10	-	0.107	0.0946	0.88	90.2	100.5
	+	0.09	0.101	1.13	89.9	96.5
100	-	0.0949	0.117	1.24	89.1	95.3
	+	0.0752	0.0927	1.23	86.9	97

After IV administration of FC-12738 at 1 mg/kg in male beagle dogs, FC-12738 showed Cl of 5.85 ± 0.940 mL/min/kg, $T_{1/2}$ at 0.680 ± 0.0504 h (Table 3). V_{dss} was 0.302 ± 0.0636 L/kg, the area under the plasma concentration time curve from time zero to AUC_{0-last} value was 2850 ± 439 ng·h/mL. Following single SC administration of FC-12738 at 5 mg/kg in male beagle dogs, AUC_{0-last} value of FC-12738 was 16100 ± 2510 ng·h/mL, the C_{max} value was 7300 ± 1050 ng/mL, while T_{max} was reached at 0.833 ± 0.289 h. The SC bioavailability was 112% at 5 mg/kg. All animals tolerated FC-12738 at the dosing levels during the entire course of the study. No adverse effect was observed during the in-life phase of the study.

Following SC administration of FC-12738 at 1, 5 and 25 mg/kg in male beagle dogs, the area under the plasma concentration time curve from time zero to AUC_{0-last} values

Table 2: Mean pharmacokinetic parameters of FC-12738 in male SD rats

PK parameters	IV	SC
	2 mg/kg, single	10 mg/kg, single
	Mean	Mean
C ₀ or C _{max} (ng/mL)*	7830	8430
T _{max} (h)	--	0.417
T _{1/2} (h)	0.318	0.68
V _{d_{ss}} (L/kg)	0.309	--
CL (mL/min/kg)	11.8	--
AUC _{0-last} (ng·h/mL)	2800	13000
AUC _{0-inf} (ng·h/mL)	2830	13000
^a Bioavailability (%)	--	91.9

*: C₀ stands for intravenous bolus administration group; C_{max} stands for subcutaneous injection group; a: bioavailability (%) was calculated with mean AUC_{0-inf} and nominal dose. "--" means not applicable.

Table 3: Mean pharmacokinetic parameters of FC-12738 in male beagle dogs

PK Parameters	IV	SC
	1 mg/kg, single	5 mg/kg, single
	Mean	Mean
C ₀ or C _{max} (ng/mL)*	4820	7300
t _{max} (h)	--	0.833
t _{1/2} (h)	0.68	0.884
V _{d_{ss}} (L/kg)	0.302	--
CL (mL/min/kg)	5.85	--
AUC _{0-last} (ng·h/mL)	2850	16100
AUC _{0-inf} (ng·h/mL)	2900	16200
Bioavailability (%) ^a	--	112

*: C₀ stands for intravenous bolus administration group; C_{max} stands for subcutaneous injection group. a: Bioavailability (%) was calculated with mean AUC_{0-last} and nominal dose. "--" means not applicable.

of FC-12738 were 2760 ± 120 , 16700 ± 3260 and 72800 ± 4920 ng·h/mL, respectively. The C_{max} values were 925 ± 39.7 , 7090 ± 935 and 33300 ± 4070 ng/mL, while T_{max} was reached at 1.00, 1.00 and 0.667 ± 0.289 h, respectively. The systemic exposure (AUC_{0-last} and C_{max}) of FC-12738 increased with dose proportionally as dose levels increased from 1 mg/kg to 5 mg/kg, 5 mg/kg to 25 mg/kg and 1 mg/kg to 25 mg/kg in male beagle dogs. The mean PK parameters of FC-12738 are shown in Table 4.

Protein binding Study

At 0.2, 2 and 10 μM, the binding of FC-12738 was 55.1%, 54.5% and 52.1% in CD-1 mouse plasma, 51.8%,

55.7% and 50.7% in SD rat plasma, 50.8%, 53.9% and 30.9% in beagle dog plasma, 46.2%, 46.1% and 26.4% in

cynomolgus monkey plasma, 57.2%, 53.0% and 32.7% in human plasma, respectively. The recovery of FC-12738 from dialysis wells was acceptable, ranging from 100.9% to 120.3%, indicating that the compound was stable during the dialysis process. In conclusion, FC-12738 exhibited a moderate binding to plasma proteins in CD-1 mouse and SD rat, a low to moderate binding to plasma proteins in beagle dog and human plasma, and a low binding to plasma proteins in cynomolgus monkey, at all three test concentrations (0.2, 2 and 10 μM).

Blood to plasma partitioning study

At 0.200, 2.00, and 10.0 μM, the mean KB/P values of FC-12738 were 0.48, 0.50, and 0.47 in rat blood, 0.57, 0.55, and 0.58 in dog blood whole blood, respectively. The mean erythrocyte to plasma ratio (KE/P) values were <0.21, <0.11, and <0.03 in rat blood, <0.18, 0.07, and <0.14 in dog blood whole blood, respectively. Following a 60-min incubation, the %Recovery of FC-12738 was acceptable, ranging from 89.98 to 101.03. In conclusion, FC-12738 exhibited a low partitioning into erythrocytes of rat and dog. No significant species difference or concentration-dependent partitioning was observed.

Table 4: Mean pharmacokinetic parameters of FC-12738 in male beagle dogs

PK parameters	SC		
	1 mg/kg, single	5 mg/kg, single	25 mg/kg, single
	Mean	Mean	Mean
C _{max} (ng/mL)*	925	7090	33300
T _{max} (h)	1	1	0.667
T _{1/2} (h)	0.957	0.906	2.01
AUC _{0-last} (ng·h/mL)	2760	16700	72800
AUC _{0-inf} (ng·h/mL)	2770	16800	72800

Tissue distribution study in SD rats following single subcutaneous injections

After single SC administration of FC-12738 at 10 mg/kg to male SD rats, FC-12738 was distributed to all tissues within 0.5 hour post-dose. C_{max} were observed at 0.5 hour post-dose in most of tissues. The highest tissue exposure (AUC_{0-last}) was observed in kidney (135000 ng·h/g) and lung (6840 ng·h/g), followed by small intestine, stomach, liver, large intestine, spleen and muscle. The heart, fat and brain exposures (AUC_{0-last}) were not determined due to most concentrations were below the LLOQ (40 ng/g). All animals had tolerated the compound at the dosing levels during the entire course of the study. No adverse effect was observed during the in-life phase of the study. The mean plasma concentrations (ng/mL), tissue concentrations (ng/g) and pharmacokinetic parameters of FC-12738 in male SD rats are shown in Table 5.

Plasma stability

At different time points 0, 10, 30, 60, and 120 minutes, FC-12738 showed %Remaining values of 100.0, 103.6, 100.4, 110.6 and 100.1 in CD-1 mouse plasma; 100.0, 104.4, 102.2, 112.9 and 103.6 in SD rat plasma; 100.0, 106.2, 105.1, 105.8 and 100.6 in beagle dog plasma; 100.0, 104.8, 104.9, 104.7 and 102.5 in cynomolgus monkey plasma; 100.0, 93.3, 93.6, 90.6, and 85.6 in human plasma, respectively. FC-12738 has a half-life of >372.7 minutes in CD-1 mouse, SD rat, beagle dog, cynomolgus monkey and human plasma, respectively. In conclusion, FC-12738 was stable in CD-1 mouse, SD rat, beagle dog, cynomolgus monkey and human plasma after 120 minutes incubation.

Hepatocyte stability

The half-life values of FC-12738 were >372.7, >372.7, >372.7 >372.7 and >372.7 (min) after 120 minutes incubations, corresponding to the intrinsic clearance $C_{L_{int}(hep)}$ ($\mu\text{L}/\text{min}/106$ cells) values of <1.9, <1.9, <1.9, <1.9, and <1.9; and hepatic clearance $C_{L_{int}(liver)}$ (mL/min/kg) values of <22.1, <8.7, <12.8, <6.7 and <5.2 in mouse, rat, dog, monkey, and human hepatocytes, respectively. These results indicated that FC-12738 was slowly metabolized in CD-1 mouse, SD rat, beagle dog, cynomolgus monkey and human hepatocytes.

Metabolite identification in liver microsomes

No metabolites of FC-12738 were detected and identified from human liver microsomes. Based on the identified metabolites, FC-12738 (MW = 679.4) was the major component in human liver microsomes after incubation at 37°C for 120 min. The relative abundances (UV%) of parent drug and its metabolites in human liver microsomal incubations are shown in Table 6.

Metabolite identification in hepatocytes

A total of 4 metabolites of FC-12738 were detected and identified from mouse, rat, dog, monkey, and human hepatocytes. The metabolites were assigned as M1-M4: glucose conjugation metabolites (MW=841.92, P + C₆H₁₀O₅). Based on the identified metabolites, the major metabolic pathway of FC-12738 in hepatocytes was proposed as glucose conjugation. The metabolites formed in incubation of human hepatocytes were detected in hepatocytes of at least one animal species. The relative abundance (UV%) of FC-

Table 5: The mean plasma concentrations (ng/mL), tissue concentrations (ng/g) and pharmacokinetic parameters of FC-12738 in male SD rats (n=3 per time point)

Time(h)\ Matrix	Concentration (ng/mL or ng/g)			Parameters		
	0.5	2	8	Cmax (ng/mL or ng/g)	Tmax (h)	AUC0-last (ng·h/mL or ng·h/g)
Plasma	14200	1790	5.04	14200	0.5	14400
Liver	1270	616	269	1270	0.5	4190
Kidney	28500	17300	14300	28500	0.5	135000
Stomach	3370	555	96.7	3370	0.5	4760
Lung	5330	752	109	5330	0.5	6840
Small Intestine	2060	775	188	2060	0.5	4970
Large Intestine	2150	430	226	2150	0.5	4040
Heart	2150	365	ND	ND	ND	ND
Muscle	1490	191	46.4	1490	0.5	1930
Spleen	1490	345	239	1490	0.5	3280
Fat	1070	103	ND	ND	ND	ND
Brain	128	ND	ND	ND	ND	ND

ND: not determined as more than half (>50%) of the individual values were not quantifiable

Table 6: The relative abundance of metabolites and FC-12738 after incubated with human liver microsomes

Metabolite Code	[M+H] ⁺ m/z	RT (min)	Relative Abundance (MS Peak Area %Total) ^a	Metabolic Pathways
			human	
FC-12738	680.3721	21.47	100	NA, Parent Drug

RT: Retention time of LC-MS; NA: Not applicable. A $\%Total = \frac{\text{Peak Area of a Related Component}}{\text{Peak Area of Total Related Components}} \times 100\%$

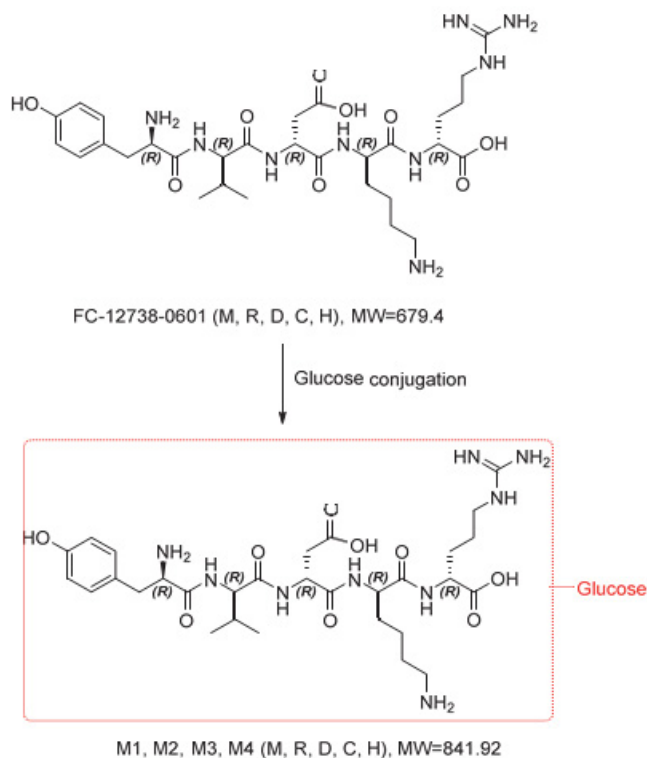


Figure 1: Proposed metabolic pathway of FC-12738 in hepatocytes, plasma, urine, bile, and feces.

Table 7: The metabolic changes and relative abundance of FC-12738 and its detected metabolites in hepatocytes

Metabolite Code	[M+H] ⁺ m/z	RT(min)	Relative Abundance (UV%) ^a					Metabolic Pathways
			Mouse	Rat	Dog	Monkey	human	
*M1	842.4268	20.24	0.33	0.23	0.18	0.27	0.21	Glucose conjugation (P+ C6H10O5)
*M2	842.4252	21.07	3.76	2.87	2.85	2.6	2.66	Glucose conjugation (P+ C6H10O5)
FC- 12738	680.3719	21.22	88.92	87.56	80.76	89.24	91.82	NA
*M3	842.4255	22.63	5.15	3.18	4.17	3.14	3.28	Glucose conjugation (P+ C6H10O5)
*M4	842.4258	24.82	1.84	1.31	1.48	1.19	1.28	Glucose conjugation (P+ C6H10O5)

P: parent drug; NA: Not applicable; *: metabolites were detected in NC (without hepatocytes); $\%Total = \frac{\text{Peak Area of a Related Component}}{\text{Peak Area of Total Related Components}} \times 100\%$

12738 and each putative metabolites were provided in Table 7. The proposed major metabolic pathway in hepatocytes of FC-12738 was shown in Error! Reference source not found.

Metabolism of FC-12738 in SD rat plasma

In addition to unchanged FC-12738 (MW = 679.37), a total of 3 metabolites of FC-12738 were detected and identified in male SD rat plasma. The metabolites were assigned as: M2-M4: glucose conjugation metabolites (MW = 841.92, P + C₆H₁₀O₅). The relative abundance of FC-12738 and its metabolites is shown in Table 8. Based on the identified metabolites, the proposed formation pathway of major metabolites of FC-12738 in male SD rat plasma was glucose conjugation. The proposed formation pathways of major metabolites of FC-12738 in SD rat plasma are shown in Error! Reference source not found.

Metabolism of FC-12738 in SD rat urine, feces and bile

A total of 2 metabolites of FC-12738 were detected and identified in rat urine, bile and feces. The metabolites were assigned as: M3 and M4: glucose conjugation metabolites (MW = 841.92, P + C₆H₁₀O₅). The relative abundance of FC-12738 and its metabolites shown as in Table 9. Based on the identified metabolites, the major metabolic pathway of FC-12738 in rat urine, bile and feces was proposed as glucose conjugation. The proposed major metabolic pathways of FC-12738 in rat urine, bile and feces are presented in Figure 1.

Excretion study in intact SD rats following single subcutaneous administrations

Following a single SC administration of FC-12738 at 10 mg/kg, the mean drug recovery of FC-12738 from urine and feces in the male intact rats at 168 hours post-dose were 86.7±6.25% and 2.19±1.22%, respectively, of the nominal dose. The total recovery rate was 88.9±5.76% of the dosage, and urine was the main excretion route.

Drug recovery of FC-12738 from bile in the male bile-duct-cannulated (BDC) rats at 72 hours post-dose was 0.336±0.260% of the nominal dose. All animals had tolerated the compound at the dosing levels during the entire course of the study. No adverse effect was observed during the in-life phase of the study. The accumulative dose recovery (%) of FC-12738 in bile, urine and feces of male SD rats following single SC administration of FC-12738 at 10 mg/kg is shown in

CYP450 inhibition assay

The remaining activities of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A (with midazolam and testosterone as probe substrate, respectively) treated with FC-12738 were no less than 97.4%, 99.9%, 99.0%, 96.7%, 95.9%, 95.1%, 95.1% and 97.2%, respectively. Therefore, the IC₅₀ values of FC-12738 on CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A (with midazolam and testosterone as probe substrate, respectively) were all greater than 100 μM.

Table 8: The relative MS abundance of FC-12738 and its metabolites in rat plasma

Metabolite Code	[M+2H] ²⁺	RT (min)	Male		Metabolic Pathways
			0-2 h (IV)	0-4 h (SC)	
M2	*421.7159	19.87	0.66	1.43	Glucose conjugation (P + C ₆ H ₁₀ O ₅)
FC-12738	*340.6898	19.81	97.1	96.57	NA
M3	*421.7160	22.46	2.03	1.74	Glucose conjugation (P + C ₆ H ₁₀ O ₅)
M4	*421.7158	24.56	0.2	0.26	Glucose conjugation (P + C ₆ H ₁₀ O ₅)

NA: not applicable. RT: Retention time of LC-MS; NA: Not applicable; P: Parent; *: value of [M+2H]²⁺.

$$\%Total = \frac{\text{Peak Area of a Related Component}}{\text{Peak Area of Total Related Components}} \times 100\%$$

Table 9: The relative MS abundance of FC-12738 and its metabolites in SD rat urine, bile and feces

Metabolite Code	[M+2H] ²⁺	RT (min)	Male			Metabolic Pathways
			Urine	Feces	Bile	
FC-12738	*340.6896	20.03	99.93	100	100	NA
M3	*421.7161	22.49	0.06	ND	ND	Glucose conjugation (P + C ₆ H ₁₀ O ₅)
M4	*421.7153	24.66	0.01	ND	ND	Glucose conjugation (P + C ₆ H ₁₀ O ₅)

NA: not applicable. RT: Retention time of LC-MS; NA: Not applicable; P: Parent; *: value of [M+2H]²⁺.

$$\%Total = \frac{\text{Peak Area of a Related Component}}{\text{Peak Area of Total Related Components}} \times 100\%$$

Table 10: Accumulative Dose Recovery (%) of FC-12738 in Bile, Urine and Feces of Male SD rats Following Single subcutaneous injection administration of FC-12738 at 10 mg/kg

Matrix	Collection Time (h)	Male	
		Mean	SD
Urine	0	0	--
	4	56.2	26.2
	8	78.2	8.68
	24	84	6.22
	48	86.1	6.51
	72	86.4	6.35
	96	86.4	6.31
	120	86.6	6.25
	144	86.7	6.19
	168	86.7	6.25
Feces	0	0	--
	4	0.00168	0.00292
	8	0.00239	0.00414
	24	1.74	0.962
	48	2.08	1.2
	72	2.14	1.21
	96	2.16	1.22
	120	2.18	1.22
	144	2.19	1.23
Matrix	Collection Time (h)	Male	
		Mean	SD
Total	168	2.19	1.22
	0	0	--
	4	56.2	26.2
	8	78.2	8.68
	24	85.8	5.85
	48	88.2	6
	72	88.5	5.81
	96	88.6	5.76
	120	88.7	5.71
	144	88.9	5.71
	168	88.9	5.76
Bile	0	0	--
	4	0.284	0.279
	12	0.314	0.27
	24	0.324	0.271
	48	0.331	0.264
	72	0.336	0.26

CYP time-dependent inhibition assay

The fold of IC50 shift for CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A (midazolam as substrate) and CYP3A (testosterone as substrate) could not be calculated because the IC50 values were greater than 100 μM for the pre-incubation with or without NADPH. Using an IC50 shift greater than or equal to 1.5-fold as the cutoff for time-dependent inactivation, it was concluded that FC-12738 showed no time-dependent inhibition on any of the CYP isozymes tested.

CYP induction assay

The released LDH data show that FC-12738 at 0.100, 1.00, 3.00, 10.0 and 15.0 μM has no cytotoxicity in all three donors. The data from enzyme activities levels and gene expression levels in all three donors show that FC-12738 at the concentrations ranging from 0.100 to 15.0 μM is not considered as an inducer for CYP1A2, CYP2B6 and CYP3A4. The metabolic stability data show that following incubation with human hepatocytes for 24 hours, the % remaining for FC-12738 at concentrations tested were found to be 107.4, 105.0, 47.1, 69.1, 86.4 in Donor 1, 89.2, 112.4, 61.9, 68.7, 89.3 in Donor 2, and 93.8, 100.3, 55.0, 61.0, 91.7 in Donor 3, respectively.

Assessment of FC-12738 on the activity of P-gp and BCRP in the Caco-2 cells

Under the conditions tested, the %P gp Activity values of Caco-2 cells treated with FC-12738 were no less than 93.9, therefore, the IC50 value of FC-12738 on P-gp was greater than 100 μM. Under the conditions tested, the %BCRP Activity values of Caco-2 cells treated with FC-12738 were no less than 93.3, therefore, the IC50 value of FC-12738 on BCRP was greater than 100 μM.

Assessment of FC-12738 on the activity of OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1 and MATE2-K transporters

The %VC values of HEK293-OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1, and MATE2-K cells treated with FC-12738 were no less than 99.4, 89.6, 98.4, 98.8, 99.6, 91.3, and 62.6, respectively. Therefore, the IC50 values of FC-12738 on OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1, and MATE2-K were greater than 100 μM.

Comparison of TP5 and FC-12738 levels in plasma and brain in male CD-1 mice after a single IP dose

The plasma and brain levels of TP5 and FC-12738 were compared in male CD-1 mice after an IP injection of each peptide at a concentration of 10mg/ml. Vehicle formulations for the two peptides were identical. In the first 2 hours after injection, the levels of FC-12738 in plasma and brain were substantially higher than that of TP5 (Table 11). These

Table 11: Levels of TP5 and FC-12738 in plasma and brain of CD-1 mice

Analyte	Matrix	Time (h)	Plasma or Tissue Homogenate Conc (ng/ml) n=3			Mean (ng/ml)	Mean Tissue (ng/g)
FC-12738	Plasma	0.5	2230	1790	2260	2093	N/A
		1	885	1320	1420	1208	
		2	543	592	745	627	
		4	97.9	74.6	79.8	84.1	
		8	19.1	17.5	13.4	16.7	
	Brain	0.5	17.4	17.6	14.1	16.4	65.5
		1	8.28	10.1	8.41	8.93	35.7
		2	6.96	5.73	7.47	6.72	26.9
		4	1.76	1.22	1.06	1.35	5.39
		8	ND	ND	ND	ND	ND
TP5	Plasma	0.5	144	136	115	132	N/A
		1	80.1	107	85.7	90.9	
		2	56.5	67.2	54.8	59.5	
		4	53.4	42.2	51.3	49	
		8	28.7	30.6	23.6	27.6	
	Brain	0.5	2.34	2.17	3.18	2.56	10.3
		1	1.69	1.78	2.2	1.89	7.56
		2	1.64	1.28	1.22	1.38	5.52
		4	1.16	1.03	1.08	1.09	4.36
		8	ND	ND	ND	ND	ND

findings are consistent with the idea that FC-12738 has a substantially longer half-life in plasma than TP5, allowing for greater uptake in brain.

Conclusions

In regard to absorption, FC-12738 showed low permeability across the Caco-2 cell monolayer and was a poor or non-substrate for efflux transporters. Following single SC administration of FC-12738 at 10 mg/kg in male SD rats and at 5 mg/kg in male beagle dogs, FC-12738 showed great bioavailability. And in single rising dose study in beagle dogs at 1, 5 and 25 mg/kg, the systemic exposure (AUC_{0-last} and C_{max}) of FC-12738 increased with dose proportionally as dose levels increased from 1 mg/kg to 5 mg/kg, 5 mg/kg to 25 mg/kg and 1 mg/kg to 25 mg/kg in male beagle dogs.

In regard to distribution in plasma, FC-12738 exhibited a moderate binding to plasma proteins in CD-1 mouse and SD rat plasma, a low to moderate binding to plasma proteins in beagle dog and human plasma, and a low binding to plasma proteins in cynomolgus monkey, at all three test concentrations tested (0.2, 2 and 10 μ M). FC-12738 exhibited a low partitioning into erythrocytes of rat and dog. No significant species difference or concentration- dependent partitioning was observed.

In the tissue distribution study in SD rats following single

SC, the highest tissue exposure (AUC_{0-last}) was observed in kidney (135000 ng·h/g) and lung (6840 ng·h/g), followed by small intestine, stomach, liver, large intestine, spleen and muscle. The heart, fat and brain exposures (AUC_{0-last}) were not determined due to most concentrations were below the LLOQ (40 ng/g).

In regard to drug metabolism, FC-12738 was stable in CD-1 mouse, SD rat, beagle dog, cynomolgus monkey and human plasma after 120 minutes incubation and slowly metabolized in CD-1 mouse, SD rat, beagle dog, cynomolgus monkey and human hepatocytes. After incubation with plasma from CD-1 mouse, SD rat, beagle dog, cynomolgus monkey and human hepatocytes for 240 min, a total of 4 metabolites of FC-12738 were detected and identified. The metabolites were assigned as M1-M4: glucose conjugation metabolites (MW = 841.92, P + C₆H₁₀O₅). Based on the identified metabolites, the major metabolic pathway of FC-12738 in hepatocytes was proposed as glucose conjugation. After incubation with human liver microsomes, no metabolites of FC-12738 were detected. Following a single IV dose of 2 mg/kg and SC dose of 10 mg/kg, 3 glucose conjugation metabolites (M2, M3, M4) were detected in rat plasma. Following single SC administration at the dose of 10 mg/kg FC-12738, 2 glucose conjugation metabolites (M3, M4) were detected in rat urine. No metabolites were detected in bile and feces.

In regard to drug excretion, following a single SC administration of FC-12738 at 10 mg/kg, the mean drug recovery of FC-12738 from urine and feces in the male intact rats at 168 hours post-dose were 86.7±6.25% and 2.19±1.22%, respectively, of the nominal dose. Drug recovery of FC-12738 from bile in the male bBDC rats at 72 hours post-dose was 0.336±0.260% of the nominal dose. These data indicate that urine was the main excretion route.

To examine potential drug-drug interactions we examined CYP450. The IC₅₀ values of FC-12738 on CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A (with midazolam and testosterone as probe substrate, respectively) were all greater than 100 μM and showed no time-dependent inhibition on any of the CYP isozymes tested. In the CYP450 induction study, FC-12738 at the concentrations ranging from 0.100 to 15.0 μM did not induce CYP1A2, CYP2B6 and CYP3A4. FC-12738 showed no inhibitory potential on the activity of efflux transports (P-gp and BCRP), the IC₅₀ values of FC-12738 were both greater than 100 μM. Similarly, the IC₅₀ values of FC-12738 were all greater than 100 μM for transporters OATP1B1/3, OAT1/3, OCT2, MATE1/2-K.

Collectively, these findings indicate that FC-12738 has improved stability over TP5 with low potential for adverse reactions. A study by Li et al reported that TP5 binds to TLR2 with a KD of 1.57 μM, which equates to a fluid concentration of 1.060 ng/ml [1]. Assuming that FC-12738 has a similar affinity for TLR2, the levels of the peptide in brain were well above the KD for binding the target for at least 2 hours post-injection. These findings suggest that FC-12738 could be utilized to manipulate neuroinflammation in neurodegenerative disease. Additional studies are required to determine the affinity of FC-12738 for TLR and its efficacy in target engagement in vivo.

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