1. Assessment of GE-179-mediated effects on NMDA receptor activation using an *in vitro* functional assay

1.1 Summary

- An NT2 human teratocarcinoma cell line was differentiated into neuron-like cells expressing functional NMDA receptors.
- A fluorescent dye based assay was used to monitor activation of NMDA receptors (by measuring Ca^2 influx over time).
- GE-179 can inhibit NMDA receptor activation in a dose dependant manner similar to MK801, the benchmark NMDA receptor specific inhibitor.
- The mechanism of GE-179-mediated inhibition does not involve competition with the glutamate binding site, as L-Glutamate levels 1000 fold higher than GE-179 did not alter its inhibitory effect.

1.2 Introduction

The purpose of this study was to assess the effects of GE-179 on functional aspects of the NMDA receptor. The experiments were based on an in vitro cell system of NMDA receptor expression, where the effect of GE-179 on receptor activation could be compared with that of the benchmark compound MK801.

1.3 Materials & Methods

The NT2 cell system has been previously described in the literature (*1-3*), and minor protocol modifications are described in the methods below.

Cell line and chemicals

- NT2 embryonic teratoma cells were obtained frozen from ECACC and stocks were kept in liquid nitrogen.
- 10 mM stock solution of GE-179 was made in DMSO was kept frozen and was used within 48hr.

Key commercially available materials were as listed in Supplemental Table 1.

Protocol

The general protocols used in these studies were as follows:

Cell culture

- Cell culture: NT2 cells were seeded at 2x106 cells/ 75cm2 flask in 20ml Dulbecco's Modified Eagle Medium (DMEM) in the presence of 1μM retinoic acid. Medium was changed every 3-4 days. Following 4 weeks of differentiation, cells were detached using Accutase for further analysis.
- Seeded in either 6, 48 or 24 well plates at a density of 5x104 cells/ cm2 in DMEM containing 1μM cytosine arabinofuranoside, 10μM 5-fluoro-2-deoxyuridine, 10μM uridine. Medium changed every 3-4 days.

Detection of calcium ion influx

- Dissolved one vial of Fluo4 dye (50μg) in 10μl DMSO. Dilute to 25ml Hank's buffered saline (HBS), giving a final concentration of 2μM Fluo4
- Cells washed cells twice with HBS
- Added ~2.5ml Fluo4 mix per well (6 well plate)
- Incubated at 370C for 1hr
- Washed x3 with HBS
- Added ~2.5ml HBS per well
- Incubated at room temperature for 30min
- Activation monitoring of cells:
- Read baseline fluorescence levels in Tecan fluorescence plate reader (excitation/emission wavelengths 485/535nm)
- Added required ligand
- Read fluorescence

FACS analysis

- All centrifugations 5min @ 2000rpm
- Harvested cells with accutase,

- Centrifuged 3x106 cells
- Fixed and permealised cells using SantaCruz Fixation and Permebilization buffer kit according to manufacturer's instructions (using 1ml/106 cells)
- Centrifuged and resuspend in 5ml ice cold Santa Cruz Wash buffer. Repeat twice
- Resuspended in 300µl Wash buffer
- Added either NMDA-PE antibody or IgG-PE Ab, incubate for 1 hr, dark, RT
- Resuspended in 1ml of ice cold wash buffer, centrifuge. Repeated twice.
- Resuspended in 500µl wash buffer, analyze within 24hr.

Statistical analysis

Data were plotted using Prism and plotted as mean values +/-SD. A minimum of 2 replicates was used for each point.

1.4 Results

NMDA receptor expression in NT2 cells

A cell based system of NMDA receptor expression was established by long term differentiation of NT2 cells in the presence of retinoic acid. This system has been described previously in the literature (*1-3*). Following 3.5-4 week differentiation period the cells were expressing NMDA receptor as assessed by FACS analysis with an anti-NMDA receptor antibody (see Supplemental Figure 1). Cells were then usable for 1-2 weeks.

Detection of intracellular calcium ion levels

Fluo4 is a calcium ion detecting dye, which fluoresces strongly at the 535nm range when bound to Ca^{2+}. NT2 cells were incubated with Fluo4 according to the manufacturer's protocol at 2µM final concentration. This enabled detection of changes in intracellular calcium ions by measuring fluorescence in a plate reader format. A test with 1mM Calcium ionophore (which triggers non specific internalization of calcium ions) demonstrated the dye's sensitivity (see Supplemental Figure 2). Minimal variation in basal levels of Fluo4 signal was observed.

Monitoring of NMDA receptor activation

NMDA receptor activation in NT2 cells was caused by stimulation using L-Glutamate (as previously documented in literature, see references) and monitored by measuring Ca2+ influx using Fluo4. Supplemental Figure 3 shows that an increase of >60% in Fluo4 fluorescence is achieved upon NMDA receptor stimulation and this has been monitored over a 300 second period. To demonstrate specificity for the NMDA receptor the experiment was repeated in the presence of increasing concentrations of MK801, a potent NMDA receptor specific inhibitor. Supplemental Figure 3 shows that this leads to dose dependant decrease in Fluo4 signal and demonstrates that changes in Fluo4 signal can be attributed to NMDA receptor activation.

Effect of GE-179 on NMDA receptor activation

The experiments described above were repeated using GE-179 as an NMDA receptor inhibitor. The results in Supplemental Figure 4 show that GE-179 can similarly inhibit L-glutamate mediated activation of NMDA receptor in a dose dependant manner.

GE-179 competition with glutamate binding site

In order to rule out that GE-179 inhibits NMDA receptor through competition with the glutamate binding site, the following experiment was carried out. Cells were stimulated in the presence of 50μM GE-179 and cells were exposed to increasing concentrations of L-Glutamate, up to 1000 fold higher than GE-179 (up to 50mM). As shown in Supplemental Figure 5, no significant difference in the levels of inhibition was detected. This suggests that GE-179 does not compete for the Glutamate binding site on the NDMA receptor.

1.5 Conclusion

The experiments in the above studies have demonstrated that:

- NT2 cells can express functional NMDA receptors, as assessed by Ca2+ influx
- GE-179 can inhibit NMDA receptor activation in a dose dependant manner similar to MK801.
- The mechanism of GE-179-mediated inhibition does not involve competition with the glutamate binding site.

2. *In vitro* binding of [^{18}F]GE-179

2.1 Summary

Standard binding assays were performed at MDS Pharma Services to evaluate binding of GE-179 to a number of potential CNS receptors, channels and transporters. Inhibition of binding of the radiolabelled standard ligand for each target was initially assessed at 1 μM GE-179. Targets where significant binding was observed (±20% inhibition) were further assessed at 10 nM GE-179 (a concentration approximately correlating to the anticipated peak brain concentration of GE-179 following administration of [^{18}F]GE-179 to humans). With the exception of binding to the PCP site on the NMDA receptor, no other target showed significant binding at this dose (see Supplemental Table 2).

2.2 Study Objective (as given in MDA Pharma Services report)

To evaluate, in radioligand binding assays, the activity of compound E039-5 (GAE-8, PT# 1097300; [GE-179]).

2.3 Methods (as given in MDA Pharma Services report)

Methods employed in this study have been adapted from the scientific literature to maximize reliability and reproducibility. Reference standards were run as an integral part of each assay to ensure the validity of the results obtained. Assays were performed under conditions described in the accompanying "Methods" section of this report. The literature reference(s) for each assay are in the "Literature References" section. If either of these sections were not originally requested within the accompanying report, please contact us at the number below for a printout of either of these report sections.

Where presented, IC_{50} values were determined by a non-linear, least squares regression analysis using MathIQ^{TM} (ID Business Solutions Ltd., UK). Where inhibition constants (K_I) are presented, the K_I values were calculated using the equation of Cheng and Prusoff (*4*) using the observed IC_{50} of the tested compound, the concentration of the radioligand employed in the assay, and the historical values for the K_D of the ligand (obtained experimentally at MDS Pharma Services). Where presented, the Hill coefficient (n_H), defining the slope of the competitive binding curve, was calculated using MathIQ^{TM}. Hill coefficients significantly different than 1.0 may suggest that the

binding displacement does not follow the laws of mass action with a single binding site. Where IC_{50}, K_I, and/or n_H data are presented without standard error of the mean (SEM), data are insufficient to be quantitative, and the values presented (K_I, IC_{50}, n_H) should be interpreted with caution.

3. *In vivo* plasma and brain stability of [^{18}F]GE-179

3.1 Summary

Following intravenous administration of [^{18}F]GE-179 to male Wistar rats 4 radiolabelled metabolites were observed in the plasma. The amount of the total radioactivity in the plasma due to [^{18}F]GE-179 decreased from 88% at 2 min pi to 21% by 30 min pi. In contrast, in the brain 97% of the total radioactivity 2 minutes post injection was due to the parent compound, decreasing only slightly with time to 92% at 10 minutes and 84% at 30 minute post injection. Only a single radiolabelled metabolite was observed in the brain, which is likely to be a sulphoxide derivative of GE-179.

3.2 Introduction

The objective of these stability studies was to characterise the in vivo metabolism of [^{18}F]GE-179 in brain and in plasma.

3.3 Materials and methods

Equipment

All HPLC equipment is from Gilson (Anachem). The Gilson Pump delivers, at 1 ml/min, a mobile phase (A : 0.8% Triethylamine (TEA) in water (pH 7.5 with phosphoric acid; H3PO4) / B : acetonitrile) at the following gradient : 0 min (40% B), 16 min (95% B), 20 min (95% B), 20.5 min (40% B), 23 min (40% B).

The auto injector took samples from a rack, maintained at 4oC by a temperature regulator. Extracts of biological samples were injected in a 500 µl loop and eluted through a Phenomenex Column C18 (Luna 150x4.6 mm 5µm, 100 Å). UV absorbance was captured by a UV/Vis detector at 254 nm and the radioactivity in the sample, by a Radiomatic 150 TR (Flow scintillation analyzer Perkin Elmer) with a PET flow cell.

All data were captured and processed with the HPLC software Chromeleon.

Reagents

Preparations

[^{18}F]GE-179 was delivered in phosphate buffered saline with ethanol at pH 6-8. Radioactive concentrations were between 20 and 36 MBq/ml.

Solvents and water

Acetonitrile 99.9% purity (BDH)

Triethylamine (TEA) 99.6% purity (BDH)

Phosphoric acid, solution in water (Sigma)

HPLC grade Elga water 18.2 MΩ

Mobile phase preparation

8 ml of TEA were added to 992 ml of HPLC grade water. The pH was adjusted to 7.5 with H3PO4.

Methods

Biological samples collection

In vivo plasma metabolism

Each male Wistar rat (90 – 150 g) was injected with approximately 20 MBq of [^{18}F]GE-179 via a tail vein. Blood samples were collected from the femoral vein at each of the following time points post injection (pi): 2, 10 and 30 min. The animal was anaesthetised with a 50:50 mix of ketamin / xylazine at 0.1 ml/ 100g body weight (i.p.) prior to injection of the [^{18}F]GE-179.

The blood samples were centrifuged at ~2000 x g for 10 min to obtain the plasma.

The plasma was placed on wet ice and analysed by HPLC.

In vivo brain metabolism

Each male rat (90 – 150 g) was injected with approximately 20 MBq of [^{18}F]GE-179 via a tail vein. No anaesthesia was used during the study and the animals were restrained within a Perspex injection restraint. One animal was sacrificed by cervical dislocation at 2, 10 and 30 min post-injection. Immediately post-sacrifice, the brain was removed onto ice and analysed by HPLC.

Extraction, evaporation, filtration and HPLC injection

Plasma samples were extracted with 5 ml of cold acetonitrile, vortexed and centrifuged (10 min, ~3500 x g). The supernatant was evaporated to dryness using a rota vapor coupled with a water bath

at 40°C. The residue was dissolved in 0.5 ml of mobile phase (0.8% TEA in water:acetonitrile 60:40 v/v) filtered and injected into the HPLC.

The brain was washed in saline and homogenised (Waring blender) with cold acetonitrile (0.5 ml) before adding of 4.5 ml of cold acetonitrile. The brain homogenate was then processed in an identical manner to the plasma samples.

3.4 Results and discussion

Identification of the hydrophilic metabolite in brain samples

The above study identified a more hydrophilic species, in all *in vivo* brain samples, at a retention time 5.1 min that increased from 3 to 18% over 30 min. Two possible metabolites N-[2-Chloro-5-(2-fluoroethylsulfanyl) phenyl]-N'-(3-methanesulfinylphenyl)-N'-methylguanidine (sulphoxide derivative of GE-179) and N-[2-Chloro-5-(2-fluoroethanesulfinyl)phenyl]-N'-(3-methanesulfinylphenyl)-N'-methylguanidine AH113516 (disulphoxide derivative of GE-179) where identified using *in silico* metabolite prediction software (see Supplemental Figure 6). Both were synthesized, by the medicinal chemistry group, and analysed by HPLC for comparison to the *in vivo* brain samples (see Supplemental Figures 7 – 8).

3.5 Conclusions

Following intravenous administration to rats, [^{18}F]GE-179 showed a degree of metabolism, with 4 radiolabelled metabolites ingressing in the plasma with time (see Supplemental Table 3, Supplemental Figure 9). The amount of the total radioactivity in the plasma due to [^{18}F]GE-179 decreased from 88% at 2 min pi to 21% by 30 min pi.

In the brain however, [^{18}F]GE-179 was observed at high relative amounts over time with 97% parent at 2 min, 92% at 10 min and 84% at 30 min post administration. Only a single radiolabelled metabolite was observed, which is likely to be a sulphoxide derivative of GE-179 (see Supplemental Table 4, Supplemental Figure 10).

4. References

1. Zhang BX, Ma X, Zhang W, et al. Polyunsaturated fatty acids mobilize intracellular Ca2+ in NT2 human teratocarcinoma cells by causing release of Ca2+ from mitochondria. *Am J Physiol Cell Physiol.* 2006;290(5):C1321-1333.

2. Itoh T, Itoh A, Horiuchi K, Pleasure D. AMPA receptor-mediated excitotoxicity in human NT2-N neurons results from loss of intracellular Ca2+ homeostasis following marked elevation of intracellular Na+. *J Neurochem.* 1998;71(1):112-124.

3. Younkin DP, Tang CM, Hardy M, et al. Inducible expression of neuronal glutamate receptor channels in the NT2 human cell line. *Proc Natl Acad Sci U S A.* 1993;90(6):2174-2178.

4. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol.* 1973;22(23):3099-3108.

SUPPLEMENTAL TABLE 1: Key commercially available materials.

Type	Item	Supplier	Catalogue Number
Cells	NT2	ECACC	1071221
Antibodies	NMDA-PE	SantaCruz	sc-1467PE
	control-PE	SantaCruz	sc-3992
Calcium dye	Fluo-4	Invitrogen	f-14201
Compounds	MK801	Sigma	m107
	Cytosine β-D-arabinofuranoside	Sigma	C6645
	5-Fluoro-2′-deoxyuridine	Sigma	46875
	Uridine	Sigma	U3003
	retinoic acid	Sigma	r2625
Buffers	Accutase	Sigma	A6964
	DMEM	Sigma	d6546
	Fixation buffer	SantaCruz	sc-3622
	Permeabilization buffer	SantaCruz	sc-3623
	Wash Buffer	SantaCruz	sc-3624

SUPPLEMENTAL TABLE 2: *In vitro* binding of GE-179 to a number of potential CNS receptors, channels and transporters

Receptor/Channel/Transporter	Type	% inhibition at 1 μM	% inhibition at 10 nM
Adenosine A_1	Human	4	ND
Adenosine A_{2A}	Human	7	ND
Adenosine A_{2B}	Human	-15	2
Adenosine A_3	Human	10	ND
Adrenergic α_1, Non-Selective	Rat	11	ND
Adrenergic α_2, Non-Selective	Rat	16	ND
Adrenergic β, Non-Selective	Rat	10	ND
Angiotensin AT_1	Human	0	ND
Angiotensin AT_2	Human	2	ND
Calcium Channel L-Type, Dihydropyridine	Rat	-20	-18
Calcium Channel N-Type	Rat	-4	ND
Cannabinoid CB_1	Human	0	ND
Cannabinoid CB_2	Human	2	ND
Dopamine D_1	Human	6	ND
Dopamine D_{2L}	Human	-2	ND
Dopamine D_{2S}	Human	1	ND
Dopamine D_3	Human	8	ND
Dopamine $D_{4.2}$	Human	3	ND
Dopamine $D_{4.4}$	Human	0	ND
Dopamine $D_{4.7}$	Human	0	ND
Dopamine D_5	Human	-3	ND
$GABA_A$, Chloride Channel, TBOB	Rat	-6	ND
$GABA_A$, Flunitrazepam, Central	Rat	32	0
$GABA_A$, Muscimol, Central	Rat	12	ND
$GABA_B$, Non-Selective	Rat	-4	ND

Glutamate, AMPA	Rat	0	ND
Glutamate, Kainate	Rat	-14	-6
Glutamate, Metabotropic, $mGlu_5$	Human	0	ND
Glutamate, NMDA, Phencyclidine	Rat	97	54
Glycine, Strychnine-Sensitive	Rat	-1	ND
Histamine H_1	Human	-9	ND
Histamine H_2	Human	3	ND
Histamine H_3	Human	6	ND
Muscarinic, Non-Selective, Central	Rat	24	3
Neuropeptide Y Y_2	Human	-2	ND
Nicotinic Acetylcholine $\alpha 4\beta 2$, Cytisine	Rat	-6	ND
Nicotinic Acetylcholine $\alpha 7$, Bungarotoxin	Rat	4	ND
Opiate μ (OP3, MOP)	Human	-2	ND
Opiate, Non-Selective	Rat	25	-6
Potassium Channel [K_A]	Rat	-5	ND
Potassium Channel [K_{ATP}]	Human	0	ND
Potassium Channel [SK_{CA}]	Rat	12	ND
Serotonin (5-Hydroxytryptamine) 5-HT_1, Non-Selective	Rat	3	ND
Serotonin (5-Hydroxytryptamine) 5-HT_2, Non-Selective	Rat	8	ND
Serotonin (5-Hydroxytryptamine) 5-HT_3	Human	21	-2
Serotonin (5-	Guinea pig	19	ND

Hydroxytryptamine) 5-HT_4			
Serotonin (5-Hydroxytryptamine) 5-HT_{5A}	Human	27	-8
Serotonin (5-Hydroxytryptamine) 5-HT_6	Human	14	ND
Sigma σ_1	Human	35	-8
Sigma σ_2	Rat	36	8
Sodium Channel, Site 2	Rat	47	3
Transporter, Adenosine	Guinea pig	-2	ND
Transporter, Choline	Rat	1	ND
Transporter, Dopamine (DAT)	Human	19	ND
Transporter, GABA	Rat	-3	ND
Transporter, Glycine	Rat	1	ND
Transporter, Norepinephrine (NET)	Human	34	-3
Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	Human	9	ND
Vasopressin V_{1A}	Human	1	ND
Vasopressin V_{1B}	Human	5	ND

SUPPLEMENTAL TABLE 3: Relative levels of parent ([^{18}F]GE-179) and radiolabelled metabolites detected in the plasma at 2, 10 and 30 min post-injection of [^{18}F]GE-179[1]

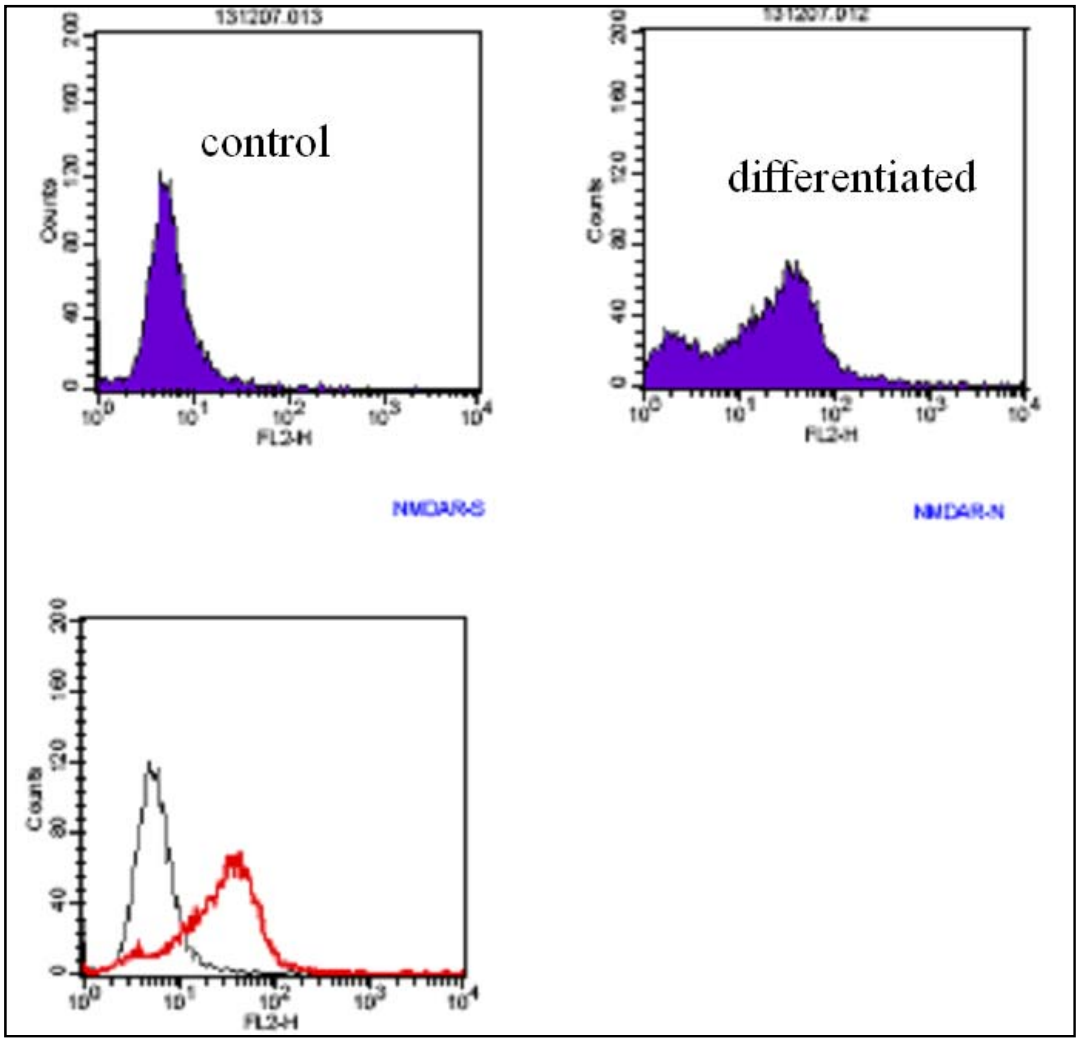
Time-point	N	Parent (% Total)	Metabolites AUC (% Total)			
			Metabolite 1 RT ~2 min	Metabolite 2 RT ~4 min	Metabolite 3 RT ~5 min	Metabolite 4 RT ~ 7.5 min
Spiked Plasma	3	100	0	0	0	0
2 min	3	88 ± 12.5	0	0	11 ± 12.5	0
10 min	4	43 ± 8	9 ± 19	21 ± 24.	15 ± 10	0
30 min	3	21 ± 13.2	27 ± 11.2	26 ± 8.5	14 ± 4.6	1.5 ± 2.3

[1] Each time point (2, 10 and 30 min) was run in triplicate and the results expressed as mean of the relative area under the curve (%AUC) with standard deviation. The spiked plasma was run in triplicate as a control.

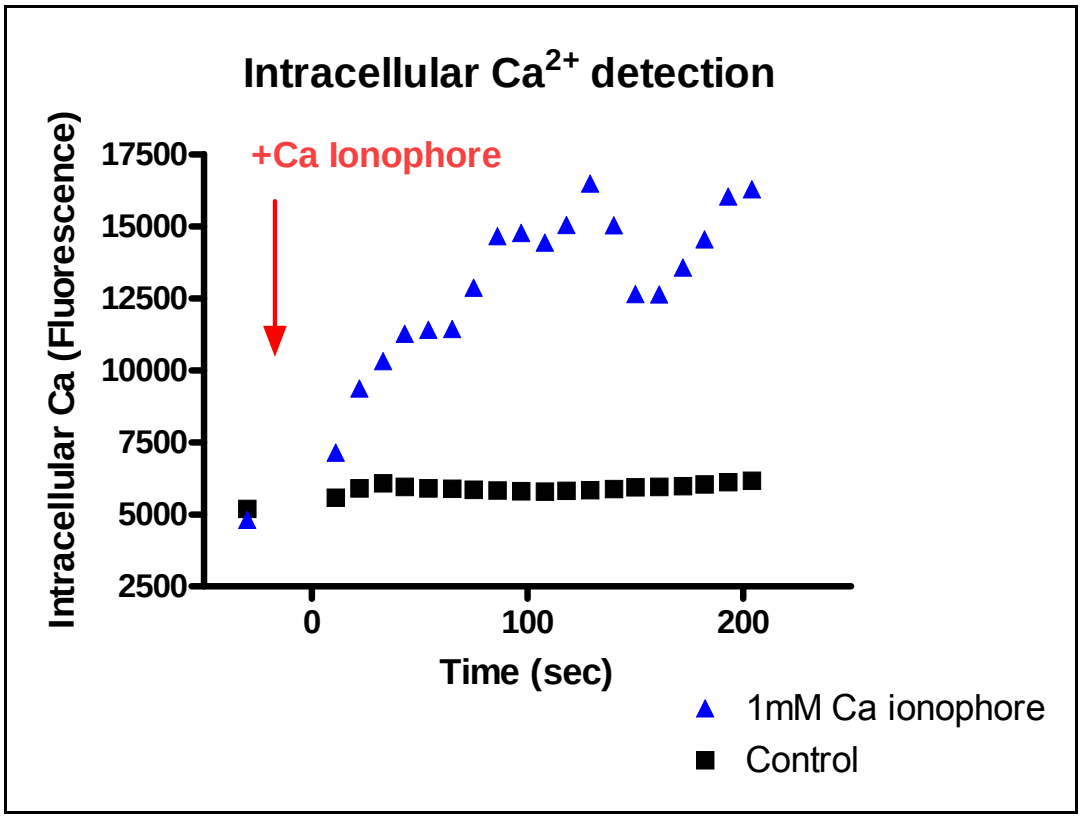
SUPPLEMENTAL TABLE 4: Relative levels of parent and radiolabelled metabolites detected in the brain at 2, 10 and 30 min post injection of [^{18}F]GE-179[2]

Time (min pi)	N	Parent AUC (% total)	Metabolite (% total)
Spiked brain	3	100	0
2 min	3	97 ± 1.5	3 ± 1.5
10 min	3	92 ± 1.7	8 ± 1.7
30 min	3	84 ± 2.0	16 ± 2.0

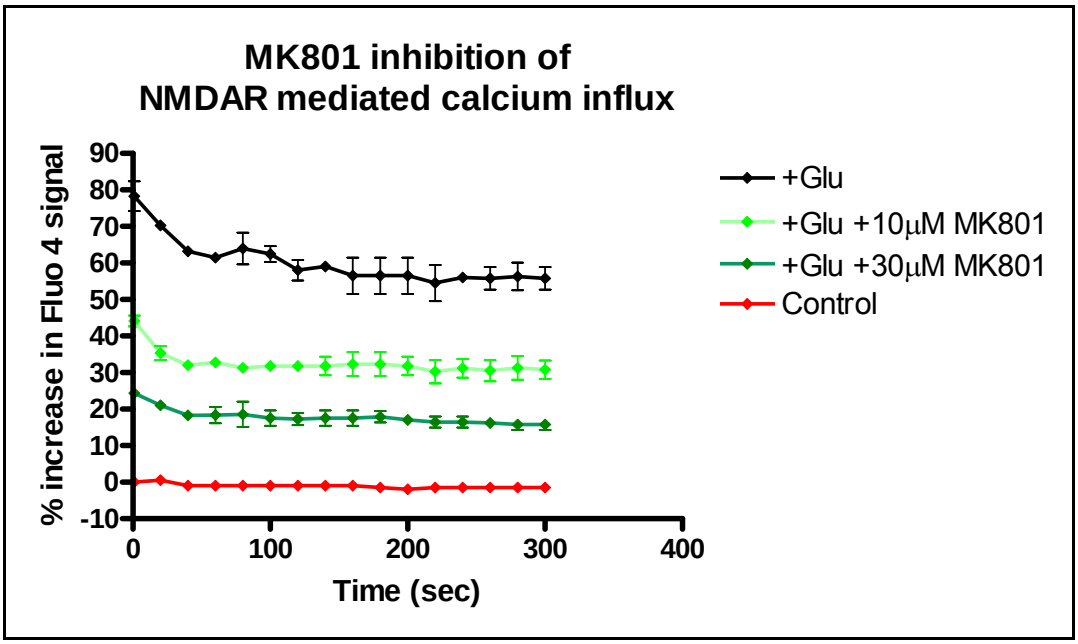
[2] Each time point (2, 10 and 30 min) was run in triplicate and the results expressed as mean of the relative area under the curve (%AUC) with standard deviation. The spiked brain was run in triplicate as a control. Only a single metabolite was observed with a retention time ~ 5 min, corresponding to metabolite 3 from Supplementary Table 3).



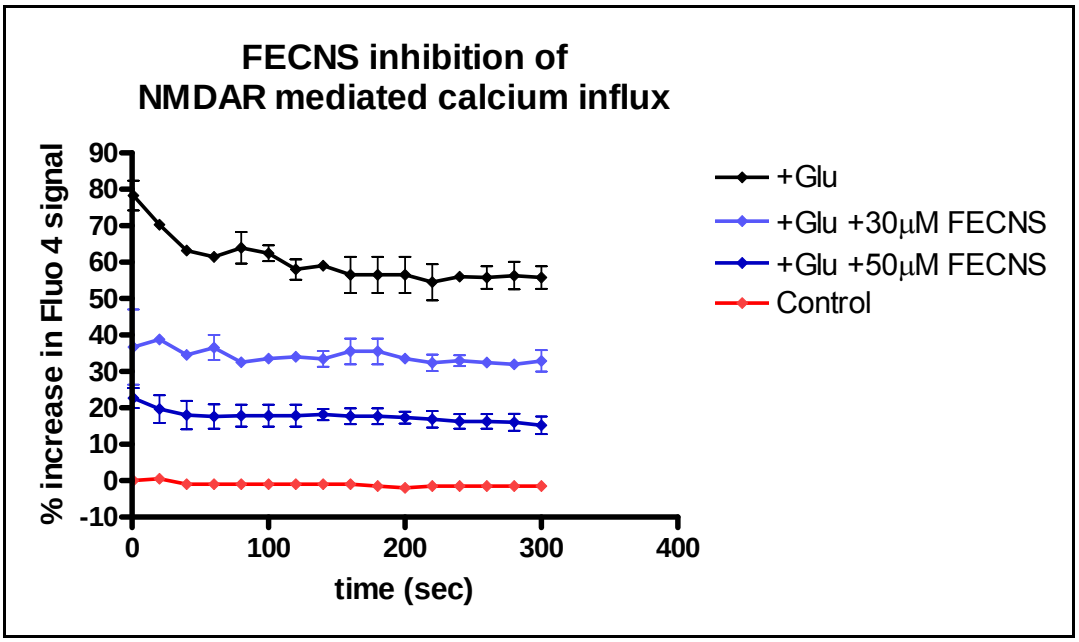
SUPPLEMENTAL FIGURE 1: Example of FACS analysis to demonstrate NMDA receptor expression by differentiated cells. Upper boxes: FL2 fluorescence (NMDA receptor) (x axis) versus cell number (y axis); bottom left box comparison of NMDA receptor expression in control versus differentiated cells.



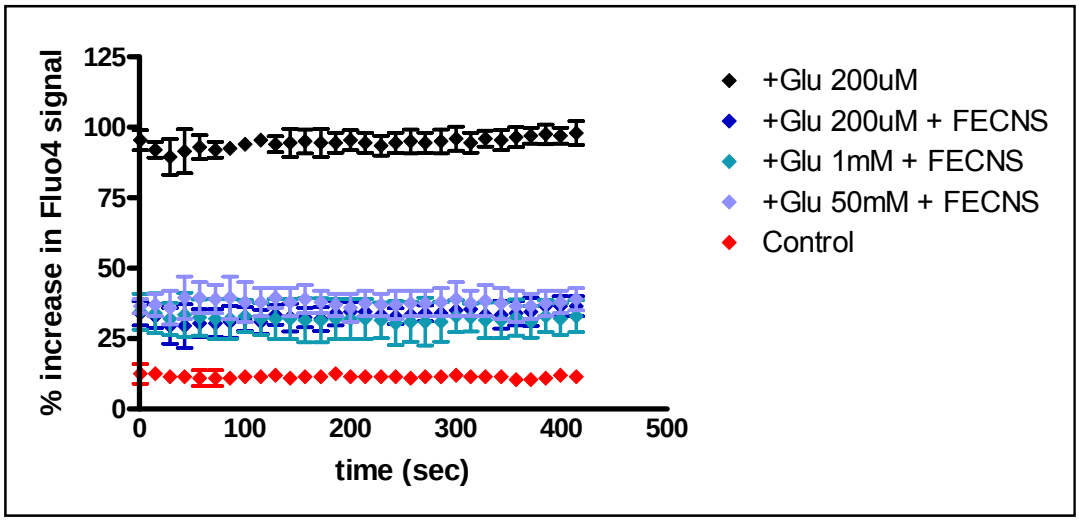
SUPPLEMENTAL FIGURE 2: Demonstration of intracellular Ca^{2+} detection using Fluo4 and calcium ionophore.



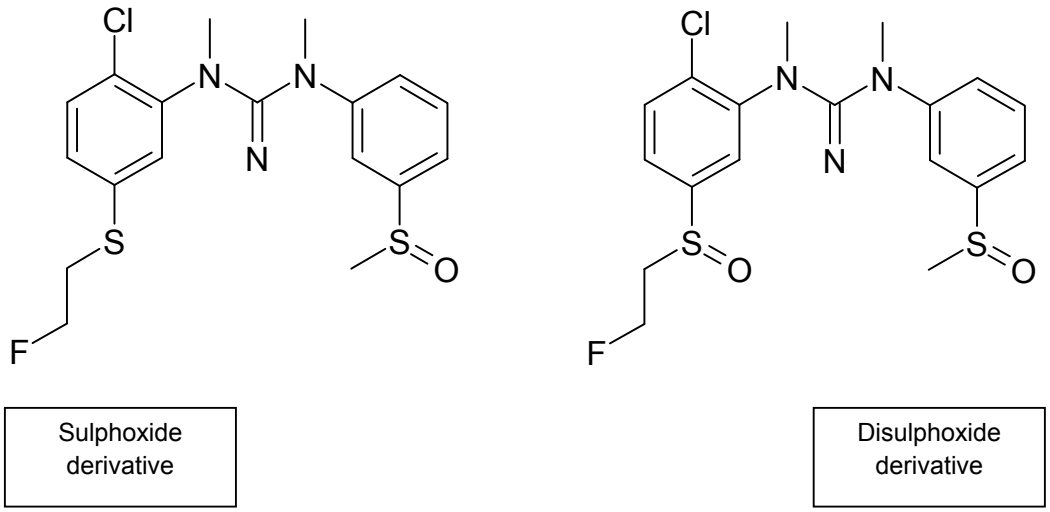
SUPPLEMENTAL FIGURE 3: MK801 inhibition of NMDA receptor-mediated calcium influx. NMDA receptor-expressing NT2 cells were incubated with 200mM L-Glutamate (+Glu) with- or without 10 or 30μM MK801. Control represents unstimulated cells in buffer containing all materials except MK801. Error bars represent standard deviation.



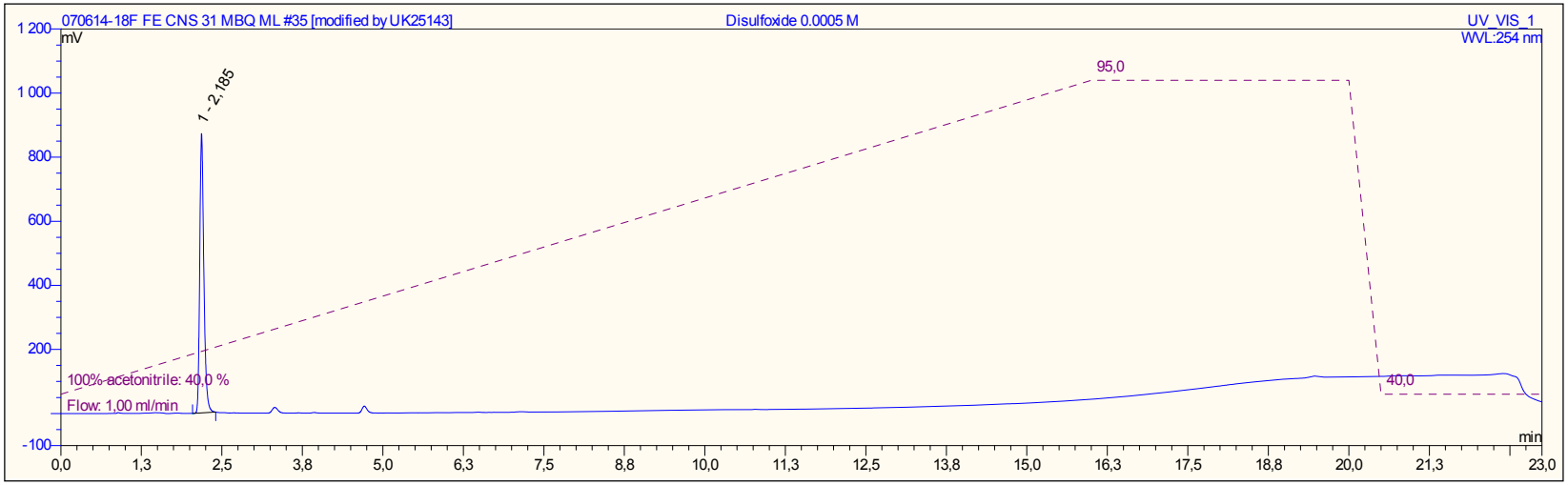
SUPPLEMENTAL FIGURE 4: GE-179 inhibition of NMDA receptor-mediated calcium influx. NMDA receptor-expressing NT2 cells were incubated with 200mM L-Glutamate (+Glu) with- or without 30 or 50μM GE-179 (referred to as 'FECNS'). Control represents unstimulated cells in buffer containing all materials except GE-179. Error bars represent standard deviation.



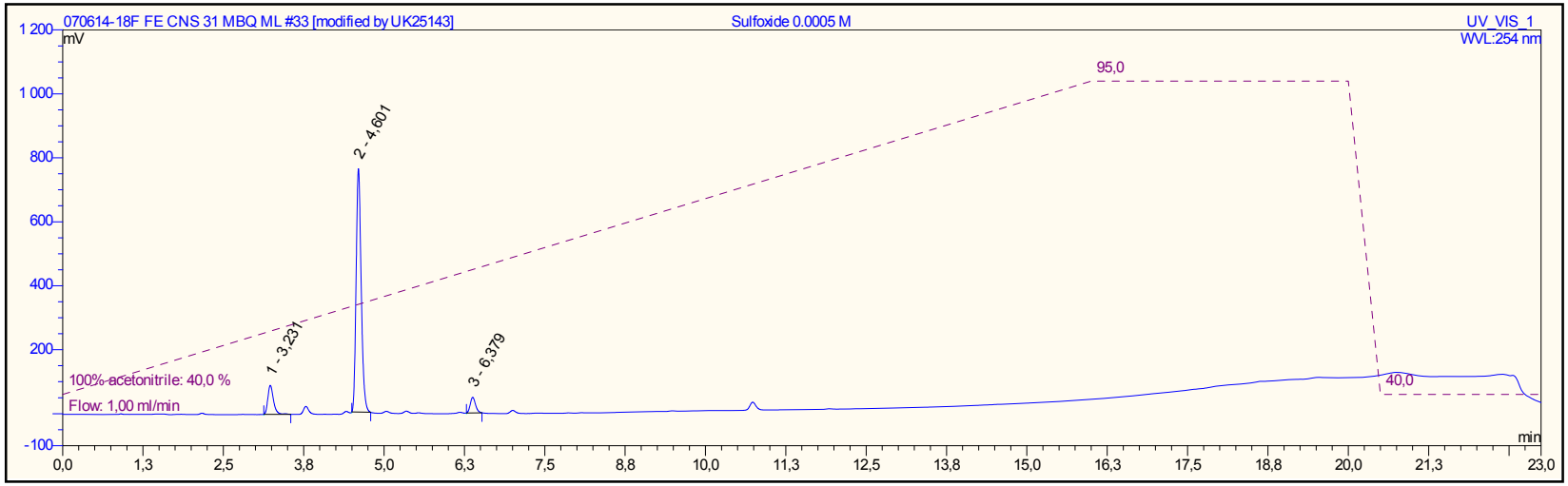
SUPPLEMENTAL FIGURE 5: GE-179 inhibition of NMDA receptor-mediated calcium influx within the presence of increasing L-Glutamate concentrations. NMDA receptor-expressing NT2 cells were incubated with 200mM L-Glutamate (+Glu) with or without 50μM GE-179 (referred to as 'FECNS'), in the presence of increasing L-Glutamate concentrations. Control represents unstimulated cells. Error bars represent standard deviation.



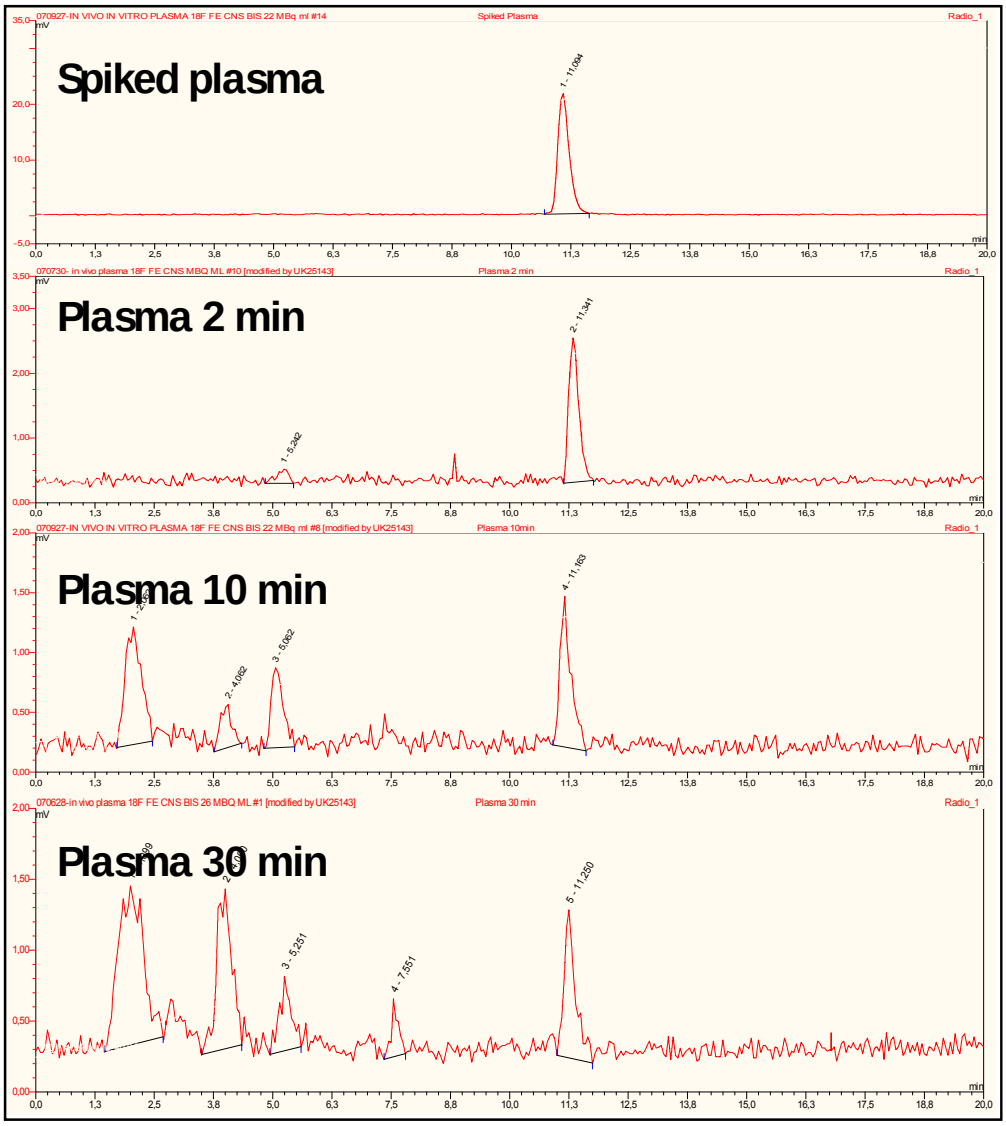
SUPPLEMENTAL FIGURE 6: Structure of the two most likely metabolite candidates for GE-179.



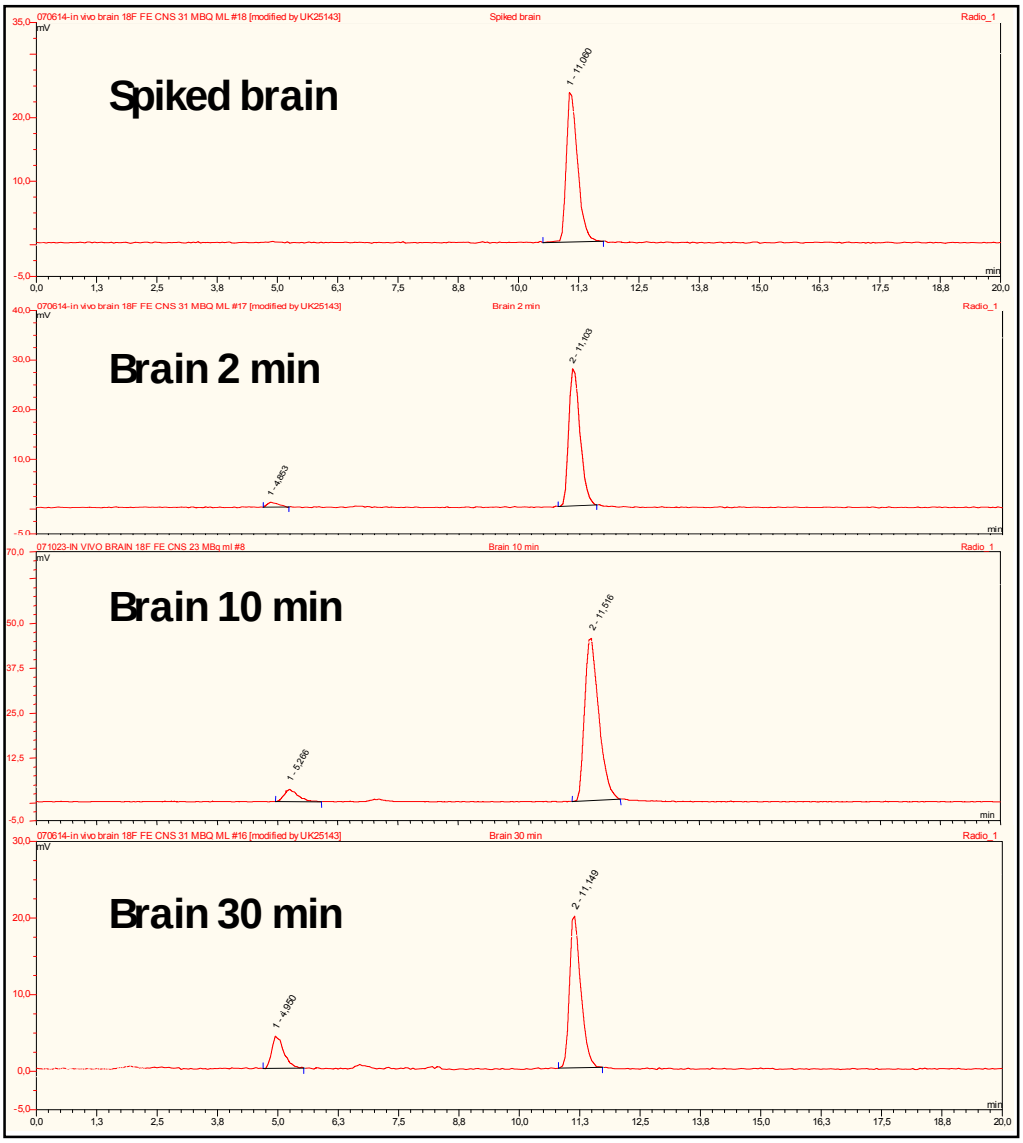
SUPPLEMENTAL FIGURE 7: HPLC analysis (UV trace) of the synthesized sulphoxide and disulphoxide at 5.10-4 M. UV trace of the disulphoxide metabolite identified a single peak running at a retention time of 2.2 min.



SUPPLEMENTAL FIGURE 8: HPLC analysis (UV trace) of the sulphoxide metabolite. Three peaks were identified, with the main peak running at a retention time of 4.6 min. Given the 0.2 min delay between the UV detector and the radio-detector the sulphoxide is likely to be the radiolabelled metabolite observed in the brain following administration of [^{18}F]GE-179.



SUPPLEMENTAL FIGURE 9: Representative traces (radioactivity detection) of plasma samples taken from animals at two minutes, 10 minutes, and 30 minutes post-injection with [^{18}F]GE-179. Spiked sample, showing only a single parent peak is given for comparison.



SUPPLEMENTAL FIGURE 10: Representative traces (radioactivity detection) of brain samples taken from animals at two minutes, 10 minutes, and 30 minutes post-injection with [^{18}F]GE-179. Spiked sample, showing only a single parent peak is given for comparison.