Synthesis and preliminary biological evaluation of a novel P2X7R radioligand [^{18}F]IUR-1601

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Abstract—The reference standard IUR-1601 ((*S*)-*N*-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-fluoroethyl)-5-oxopyrrolidine-2-carboxamide) was synthesized from *tert*-butyl (*S*)-5-oxopyrrolidine-2-carboxylate, fluoroethylbromide, and 2-chloro-3-(trifluoromethyl)benzylamine with overall chemical yield 12% in three steps. The target tracer [^{18}F]IUR-1601 ((*S*)-*N*-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-[^{18}F]fluoroethyl)-5-oxopyrrolidine-2-carboxamide) was synthesized from desmethyl-GSK1482160 with 2-[^{18}F]fluoroethyl tosylate, prepared from 1,2-ethylene glycol-bis-tosylate and K[^{18}F]F/Kryptofix2.2.2, in two steps and isolated by HPLC combined with SPE in 1-3% decay corrected radiochemical yield. The radiochemical purity was >99%, and the molar activity at end of bombardment (EOB) was 74-370 GBq/μmol. The potency of IUR-1601 in comparison with GSK1482160 was determined by a radioligand competitive binding assay using [^{11}C]GSK1482160, and the binding affinity K_i values for IUR-1601 and GSK1482160 are 4.31 and 5.14 nM, respectively.

Keywords: [^{18}F]IUR-1601 ((*S*)-*N*-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-[^{18}F]fluoroethyl)-5-oxopyrrolidine-2-carboxamide); Purinergic P2X7 receptor (P2X7R); Radiosynthesis; Competitive binding assay; Positron emission tomography (PET).

The purinergic receptor P2X ligand-gated ion channel type 7 (P2X7R) is an adenosine triphosphate (ATP)-gated ion-channel, and the overexpression of P2X7R is closely associated with neuroinflammation, which plays an important role in various neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD).[1,2] P2X7R has become a novel molecular imaging target for neuroinflammation via biomedical imaging technique positron emission tomography (PET).[3] Recently several radioligands targeting P2X7R have been developed and evaluated in animals, and the representative radioligands are shown in Figure 1.[4-10] However, preclinical evaluation indicated some of these P2X7R radioligands have significant drawbacks like short half-life, limited blood-brain barrier (BBB) penetration and/or little brain uptake. Thus an ideal P2X7R radioligand that can be used in the clinical setting to study P2X7R expression levels in neurodegenerative disorders remains to be discovered. In our previous work, we have developed and characterized [^{11}C]GSK1482160 as a P2X7R radioligand for neuroinflammation,[7,8] and clinical evaluation of [^{11}C]GSK1482160 in normal human subjects are currently underway. The half-life ($t_{1/2}$) of radionuclide carbon-11, 20.4 min, limits the imaging protocol to a maximum of about 90 min post-injection of a carbon-11 radiopharmaceutical, after that insufficient counts are available for meaningful measurements. If imaging were extended past 90 min, more complete information could be obtained regarding the concentration and localization of the P2X7R levels in the tissue. Therefore, it is attractive for us to develop

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analogs of [^{11}C]GSK1482160 which can be labeled with the radionuclide fluorine-18 ($t_{1/2}$, 109.7 min). A fluorine-18 ligand would be ideal for widespread use, which permits imaging of up to 5 h post-injection, and will result in a better match between the pharmacokinetics of binding and the physical decay of the label. To this end a series of [^{18}F]fluoroalkyl analogs of GSK1482160 will be considered as new potential P2X7R radioligands. In this ongoing study, we report here the synthesis and preliminary biological evaluation of a new P2X7R radioligand [^{18}F]IUR-1601 ((*S*)-*N*-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-[^{18}F]fluoroethyl)-5-oxopyrrolidine-2-carboxamide, [^{18}F]**3a**) (Figure 1).

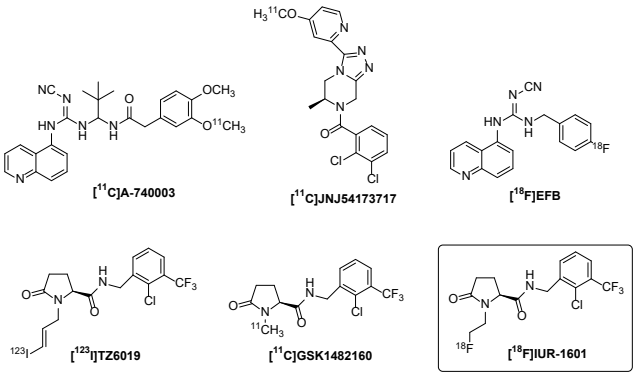
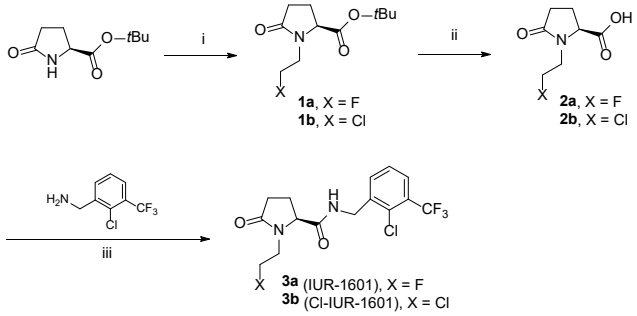


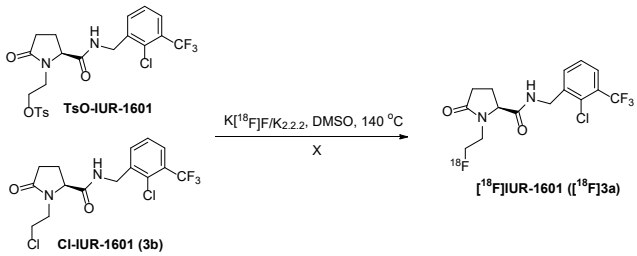
Figure 1. Radioligands for imaging of P2X7R.

Our synthetic strategy was first to conduct one-step radiosynthesis of [^{18}F]IUR-1601. The reference standard IUR-1601 ((*S*)-*N*-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-fluoroethyl)-5-oxopyrrolidine-2-carboxamide, **3a**) and its corresponding precursor Cl-IUR-1601 ((*S*)-*N*-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-chloroethyl)-5-oxopyrrolidine-2-carboxamide, **3b**) were synthesized as outlined in Scheme 1, according to the published methods.[7] *tert*-Butyl (*S*)-5-oxopyrrolidine-2-carboxylate was reacted with 1-bromo-2-fluoroethane (or 1-bromo-2-chloroethane) and NaH in *N,N*-dimethylformamide (DMF) to convert into amide *N*-alkylated product **1a** (or **1b**) in 42% and 38% yield, respectively. Subsequently, compound **1a** (or **1b**) was treated with trifluoroacetic acid (TFA) to give acid **2a** (or **2b**). Without further purification, compound **2a** (or **2b**) was undergone a coupling reaction with 2-chloro-3-(trifluoromethyl)benzylamine using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) or 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as catalyst, affording IUR-1601 (or Cl-IUR-1601) in about 28% and 27% yield, respectively.

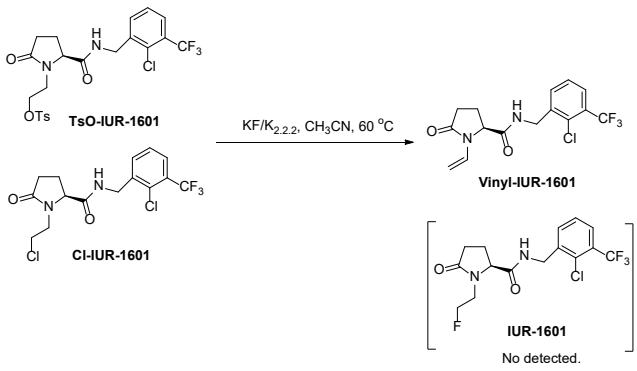
Another precursor TsO-IUR-1601 ((*S*)-2-(2-((2-chloro-3-(trifluoromethyl)benzyl)carbamoyl)-5-oxopyrrolidin-1-yl)ethyl 4-methylbenzenesulfonate) was obtained via a customer synthesis service.



Scheme 1. Synthesis of IUR-1601 (**3a**) and its precursor Cl-IUR-1601 (**3b**). Reaction conditions and regents: (i) $BrCH_2CH_2X$, NaH, DMF; 42% and 38%. (ii) TFA, CH_2Cl_2. (iii) EDAC, HOBt, CH_2Cl_2, 28% and 27%.



Scheme 2. One-step radiosynthesis of [^{18}F]IUR-1601 failed.

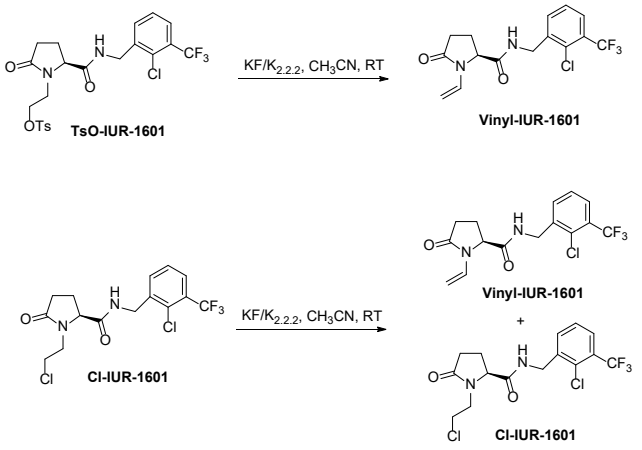


Scheme 3. Model fluorination reaction at 60 ºC.

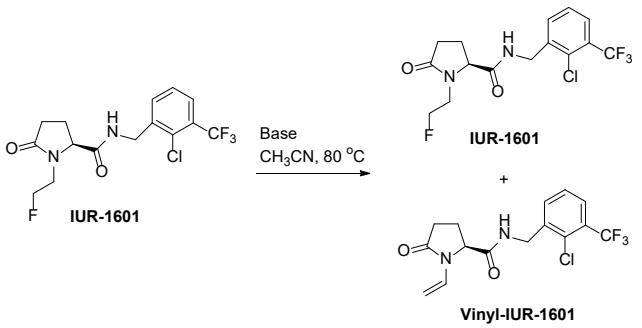
One-step radiosynthesis of [^{18}F]IUR-1601 was performed as indicated in Scheme 2 via a published method using K[^{18}F]F/Kryptofix2.2.2 and dimethyl sulfoxide (DMSO) as solvent at 140 ºC for [^{18}F]fallypride production.[11] Both Cl-IUR-1601 and TsO-IUR-1601 precursors failed to produce the radiolabeled product [^{18}F]IUR-1601. At the beginning, we suspected the reaction temperature was too high, then we conducted a model fluorination reaction without radioactivity at 60 ºC, which was monitored by analytical reverse-phase (RP) high performance liquid chromatography (HPLC). No IUR-1601 was detected, instead a compound (*S*)-*N*-(2-chloro-3-(trifluoromethyl)benzyl)-5-oxo-1-vinylpyrrolidine-2-carboxamide (vinyl-IUR-1601) was formed and detected by analytical RP HPLC, and confirmed by liquid chromatography-mass spectra (LC-MS), MS

(ESI): $[M+H]^+$ 347. The result was summarized in Scheme 3.

A few other model reactions were carried out as shown in Scheme 4 and Scheme 5. The results suggested that TsO-IUR-1601 and Cl-IUR-1601 precursors are unstable, and they would easily convert to vinyl-IUR-1601 via elimination reaction at room temperature (RT); IUR-1601 is also unstable under strong base and high temperature conditions, and it would partially form vinyl-IUR-1601 via elimination reaction as well; and thus we concluded it is impossible to use one-step radiosynthesis starting from either tosyl ethyl- or chloro ethyl- precursor to prepare [^{18}F]IUR-1601.

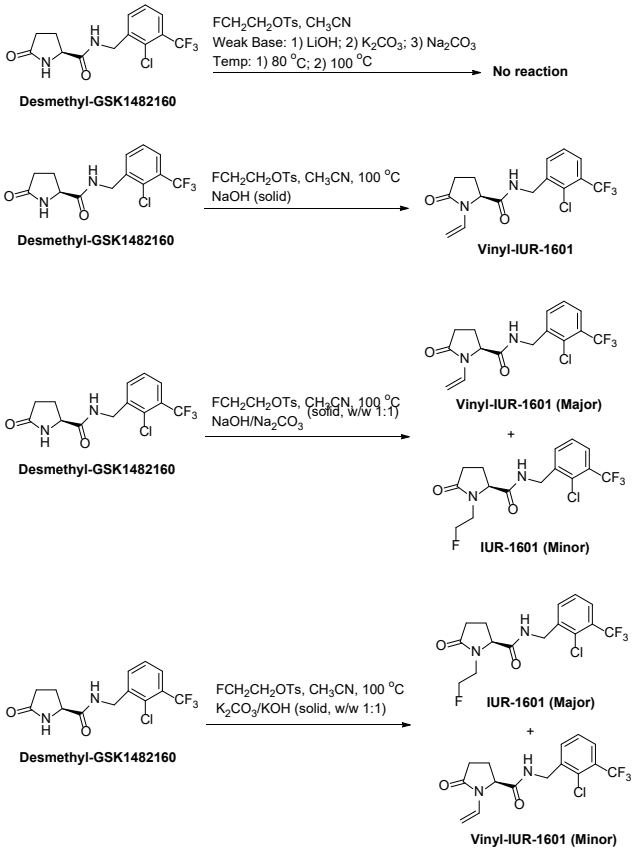


Scheme 4. Model fluorination reaction at RT.

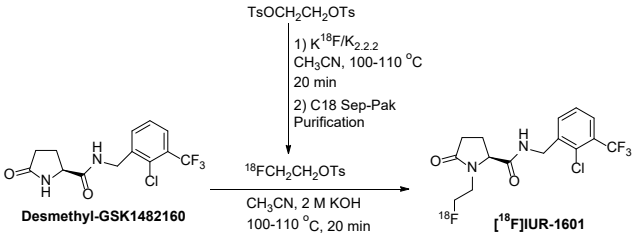


Scheme 5. Temperature and base effect on the stability of IUR-1601. The base for test included solid or liquid (1, 2, and 4 M) NaOH and KOH.

Therefore, we turned our effort to two-step radiosynthesis of [^{18}F]IUR-1601 using desmethyl-GSK1482160 precursor and radiolabeled precursor 2-[^{18}F]fluoroethyl tosylate (2-[^{18}F]FCH_2CH_2OTS)[12] prepared from 1,2-ethylene glycol-bis-tosylate ($TsOCH_2CH_2OTs$) and K[^{18}F]F/Kryptofix2.2.2. Prior to the radiosynthesis, several model reactions in small scale (0.5-1.0 mg) without radioactivity were performed to investigate the solid base and reaction temperature effect on the fluoroethylation of cyclic amide of the desmethyl-GSK1482160 precursor, as indicated in Scheme 6. All reactions were monitored by analytical RP HPLC. Based on the results we obtained, we believed that the cyclic amide is more easily deprotonated and alkylated than side chain amide; amide alkylation is a tough reaction and requires strong base and high reaction temperature conditions; and amide fluoroethylation reaction was quickly followed by an elimination reaction to form vinyl-IUR-1601 under strong reaction conditions. Further optimization of the reaction conditions provided the best method for two-step radiosynthesis of [^{18}F]IUR-1601.



Scheme 6. Temperature and base effect on the fluoroethylation of cyclic amide of the desmethyl-GSK1482160.



Scheme 7. Two-step radiosynthesis of [^{18}F]IUR-1601.

Two-step radiosynthesis of [^{18}F]IUR-1601 is indicated in Scheme 7. $TsOCH_2CH_2OTs$ was labeled with K[^{18}F]F/Kryptofix2.2.2 to form a radiolabeled precursor 2-[^{18}F]FCH_2CH_2OTs, which was simply purified by a C18 Plus Sep-Pak cartridge. Then 2-[^{18}F]FCH_2CH_2OTs was reacted with desmethyl-GSK1482160 at 100-110 ºC under basic condition (2 M KOH) for 20 min to give the target tracer [^{18}F]IUR-1601. This is a two-step two-

pot radiosynthesis. [^{18}F]IUR-1601 was isolated by the semi-preparative RP HPLC (C18 column) combined with solid-phase extraction (SPE) using a C18 Plus Sep-Pak cartridge. The radiosynthesis was performed in a self-designed automated multi-purpose [^{18}F]-radiosynthesis module.[13] This radiosynthesis module facilitated the overall design of the reaction, purification and reformulation capabilities in a fashion suitable for adaptation to preparation of human doses. Each step reaction used a V-vial. The first step used SPE (C18 Sep-Pak) for purification, and the second step used a semi-preparative C18 HPLC column for purification. C18 Sep-Pak trap/release was used for reformulation. As we previously indicated, [^{18}F]fluoroethylation of amide requires high reaction temperature and strong base; in addition, the labeled product [^{18}F]IUR-1601 is unstable under the strong reaction conditions, converting to vinyl-IUR-1601 by elimination reaction. The two-step radiosynthesis was a somewhat "hit-and-run" radiosynthesis, which resulted in very low radiochemical yield. The overall radiochemical yield for two-step radiosynthesis of [^{18}F]IUR-1601 was 1-3% decay corrected to end of bombardment (EOB) based on H[^{18}F]F. The overall synthesis time was ~2 h from EOB. The molar activity was 74-370 GBq/μmol at EOB, determined by a "spike" method using analytical HPLC at end of synthesis (EOS). The general methods to increase the molar activity have been described in our previous works,[14-16] which include rinsing the H[^{18}F]F delivery line with H_2[^{18}O]O prior to the H[^{18}F]F production, eliminating QMA Sep-Pak cartridge to trap/release the H[^{18}F]F, and using optimized beam current and beam time to produce the H[^{18}F]F. To facilitate the potential application of [^{18}F]IUR-1601 in animals, it is necessary to improve its radiochemical yield. However, the chemistry nature of [^{18}F]IUR-1601 radiosynthesis (two-step "hit-and-run") does not provide much room for the improvement. Thus, the further radiosynthesis optimization of [^{18}F]IUR-1601 will focus on the hardware and software modification of our existing home-built [^{18}F]-radiosynthesis module, such as enlarging the volume of reaction V-vial and revising the computer-controlled synthesis sequences, to minimize radioactivity loss.

Chemical purity and radiochemical purity were determined by analytical HPLC.[14-16] The chemical purity of the precursor and reference standard was >95% determined by RP HPLC through UV flow detector. The radiochemical purity of the target tracer was >99% determined by radio-HPLC through γ-ray (PIN diode) flow detector.

The analytical RP HPLC system used to monitor all organic synthetic and radiosynthetic reactions included a Prodigy (Phenomenex) 5 μm C-18 column, 4.6 × 250 mm, a gradient mobile phase (40-80%) CH_3CN/3 mM $HCOONH_4$, flow rate 1.8 mL/min; UV (270 nm) and γ-ray (PIN diode) flow detectors. Table 1 lists the retention time (t_R) data determined by this HPLC system for the compounds involved in the synthetic reactions, and their corresponding LogP and cLogP values calculated from ChemDraw Professional 15.1.

Table 1. t_R, LogP and cLogP of IUR-1601 and its analogs, $TsOCH_2CH_2OTs$ and FCH_2CH_2OTs.

Compound	LogP	cLogP	tR (min)
GSK1482160	1.88	2.26	N/D*
Desmethyl-GSK1482160	1.64	1.59	3.46
IUR-1601	2.07	2.51	4.47
TsO-IUR-1601	3.58	3.97	6.48
Cl-IUR-1601	2.59	2.63	5.08
Vinyl-IUR-1601	2.46	2.82	4.15
$TsOCH_2CH_2OTs$	3.64	2.91	7.32
FCH_2CH_2OTs	2.13	1.57	5.88

*N/D, Not determined.

GSK1482160, desmethyl-GSK1482160 and [^{11}C]GSK1482160 were synthesized in our previous work.[7]

We follow the general approach to develop a fluorine-18 radioligand. We synthesize IUR-1601 first, then we measure its *in vitro* biological activity (K_i or IC_{50}). If the *in vitro* data is encouraging, then the radiosynthesis of [^{18}F]IUR-1601 will be launched.

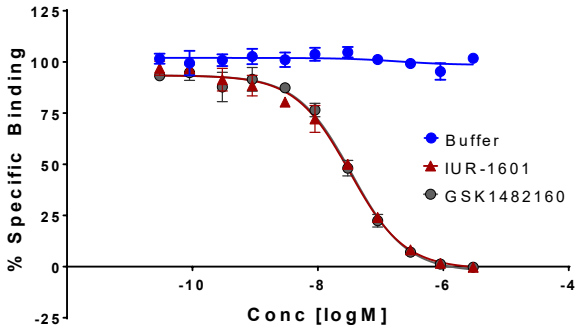


Figure 2. The result of the competitive binding assay of IUR-1601 in comparison with GSK1482160.

The preliminary biological evaluation of IUR-1601 in comparison with the known P2X7R ligand GSK1482160 was performed by a radioligand competitive binding assay using [^{11}C]GSK1482160 following the literature method.[8,17-19] The results are shown in Figure 2. [^{11}C]GSK1482160 was used as the radioligand, GSK1482160 and buffer were used as a positive control and a negative control, respectively. All data were analyzed by GraphPad Prism 7 software using a curve fitting equation to fit the K_i. The binding affinity

K_i values for IUR-1601 and GSK1482160 are 4.31 ± 0.92 nM and 5.14 ± 0.85 nM (n =3), respectively. The IC_{50} values for IUR-1601 and GSK1482160 are 7.86 nM and 7.17 nM, respectively, obtained via GraphPad nonlinear fit of data using one site completion binding analysis.

The experimental details and characterization data for compounds **1-3** and for the tracer [^{11}C]**3a,** as well as radioligand competitive binding assay are given.[20]

In summary, synthetic routes with reasonable to high yields have been developed to produce the reference standard IUR-1601 and its corresponding precursors for labeling. One-step and two-step radiosyntheses of [^{18}F]IUR-1601 have been studied. Many model reactions were designed to investigate the reasons that caused one-step synthesis of [^{18}F]IUR-1601 to fail and two-step synthesis of [^{18}F]IUR-1601 with very low radiochemical yield. A new P2X7R radioligand [^{18}F]IUR-1601 has been successfully radiosynthesized. The preliminary biological evaluation results suggest [^{18}F]IUR-1601 retains the P2X7R affinity of [^{11}C]GSK1482160.

Acknowledgments

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20. *(a). General:* All commercial reagents and solvents were purchased from Sigma-Aldrich and Fisher Scientific, and used without further purification. Melting points were determined on a MEL-TEMP II capillary tube apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II 500 MHz NMR Fourier transform spectrometer at 500 and 125 MHz, respectively. Chemical shifts (δ) are reported in parts per million (ppm) relative to an internal standard tetramethylsilane (TMS, δ 0.0) (^{1}H NMR) and to the solvent signal (^{13}C NMR), and coupling constants (*J*) are reported in hertz (Hz). LC-MS analysis was performed on an Agilent system, consisting of an 1100 series HPLC connected to a diode array detector and a 1946D mass spectrometer configured for positive-ion/negative-ion electrospray ionization. The high resolution mass spectra (HRMS) were obtained using a Waters/Micromass LCT Classic spectrometer. Chromatographic solvent proportions are indicated as volume: volume ratio. Thin-layer chromatography (TLC) was run using Analtech silica gel GF uniplates (5 × 10 cm^2). Plates were visualized under UV light. Normal phase flash column chromatography was carried out on EM Science silica gel 60 (230-400 mesh) with a forced flow of the indicated solvent system in the proportions described below. All moisture- and air-sensitive reactions were performed under a positive pressure of nitrogen maintained by a direct line from a nitrogen source. C18 Plus Sep-Pak cartridges were obtained from Waters Corporation (Milford, MA). Sterile Millex-FG 0.2 μm filter units were obtained from Millipore Corporation (Bedford, MA). [^{11}C]GSK1482160 is a routine radiotracer produced in our PET radiochemistry facility. TsO-IUR-1601 was obtained via a customer synthesis service, and ^{1}H NMR was recorded in our laboratory to confirm its quality and identity. ^{1}H NMR ($CDCl_3$): δ 2.02-2.09 (m, 1H, CH), 2.26-2.36 (m, 2H, CH_2), 2.45 (s, 3H, CH_3), 2.47-2.53 (m, 1H, CH), 3.13 (ddd, *J* = 1.5, 9.0, 15.5 Hz, 1H, CH), 3.90-3.94 (m, 1H, CH), 4.09-4.12 (m, 1H, CH), 4.16-4.20 (m, 1H, CH), 4.30-4.32 (m, 1H, CH), 4.63 (dd, *J* = 2.0, 6.0 Hz, 2H, CH_2Ph), 6.58 (t, *J* = 6.0 Hz, 1H, CONH), 7.34-7.38 (m, 3H, Ph-H), 7.61 (d, *J* = 8.0 Hz, 1H, Ph-H), 7.65 (d, *J* = 8.0 Hz, 1H, Ph-H), 7.71 (dd, *J* = 1.5, 6.5 Hz, 2H, Ph-H).
*(b). tert-Butyl (S)-1-(2-fluoroethyl)-5-oxopyrrolidine-2-carboxylate (**1a**):* *tert*-butyl (*S*)-5-oxopyrrolidine-2-carboxylate (3.74 g, 20 mmol was dissolved in DMF (30 mL) and cooled to 0 °C. Sodium hydride (1.12 g of 60% suspension in mineral oil, 28 mmol) was added to reaction mixture and stirred for 1 h. Then 1-bromo-2-fluoroethane (4.57 g, 36 mmol) was added slowly and reaction mixture was allowed to warm to RT and stirred for overnight. The solvent was evaporated in vacuo, and water was added, subsequently the aqueous layer was extracted with EtOAc (100 mL × 3), and dried over $MgSO_4$. The combined organic layer was concentrated, and the residue was purified by column chromatography on silica gel with eluent (1:99 MeOH/CH_2Cl_2) to yield colorless oily product **1a** (1.94 g, 42%), R_f = 0.68 (1:15 MeOH/CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 1.48 (s, 9H, 3 × CH_3), 2.02-2.07 (m, 1H, CH), 2.32-2.39 (m, 2H, CH_2), 2.43-2.49 (m, 1H, CH), 3.30-3.44 (m, 1H, CH), 3.81-3.91 (m, 1H, CH), 4.20 (dd, *J* = 2.5, 8.5 Hz, 1H, CH), 4.49-4.51 (m, 1H, CH), 4.58-4.60 (m, 1H, CH). MS (ESI): 232 ([M+H]$^+$, 100%).
*(c). tert-Butyl (S)-1-(2-chloroethyl)-5-oxopyrrolidine-2-carboxylate (**1b**):* Compound **1b** was prepared using the same procedure as described for the synthesis of **1a** by substituting 1-bromo-2-chloroethane for 1-bromo-2-fluoroethane. Colorless oil, yield 38%, R_f = 0.66 (1:15 MeOH/CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 1.47 (s, 9H, 3 × CH_3), 2.05-2.09 (m, 1H, CH), 2.33-2.40 (m, 2H, CH_2), 2.42-2.48 (m, 1H, CH), 3.28-3.33 (m, 1H, CH), 3.59-3.63 (m, 1H, CH), 3.69-3.74 (m, 1H, CH), 3.96 (td, *J* = 5.5, 8.5 Hz, 1H, CH), 4.25-4.27 (m, 1H, CH). MS (ESI): 248 ([M+H]$^+$, 100%).
*(d). (S)-1-(2-Fluoroethyl)-5-oxopyrrolidine-2-carboxylic acid (**2a**):* A solution of compound **1a** (578 mg, 2.5 mmol) in dichloromethane was added into TFA (1.2 mL) at RT, the reaction mixture was then stirred for 36 h. Subsequently the reaction mixture was evaporated to dry under reduced pressure. Toluene was added to the residue, and it was in turn also evaporated to give yellowish oily product, which was used for next step without further purification. MS (ESI): 176 ([M+H]$^+$, 100%); MS (ESI): 174 ([M-H]$^-$, 40%).
*(e). (S)-1-(2-Chloroethyl)-5-oxopyrrolidine-2-carboxylic acid (**2b**):* Compound **2b** was prepared using the same procedure as described for the

synthesis of **2a** by substituting **1b** for **1a**. MS (ESI): 192 ([M+H]$^+$, 100%); MS (ESI): 190 ([M-H]$^-$, 10%).

*(f). (S)-N-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-fluoroethyl)-5-oxopyrrolidine-2-carboxamide (IUR-1601, **3a**):* Compound **2a** (438 mg, 2.5 mmol) and 2-chloro-3-(trifluoromethyl)benzylamine (419 mg, 2.0 mmol) were mixed with EDAC (480 mg, 2.5 mmol) and 1-hydrobenzotrizole (HOBt, 338 mg, 2.5 mmol) in dry dichloromethane (60 mL). The reaction mixture was stirred for overnight at RT. Then the resultant mixture was washed with 2 N HCl (50 mL) and saturated aqueous $NaHCO_3$ (40 mL). The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography on silica gel with eluent (2:98 MeOH/CH_2Cl_2) to afford a white solid product **3a** (210 mg, 28%), R_f = 0.34 (1:15 MeOH/CH_2Cl_2), mp 147-149 °C. ^{1}H NMR ($CDCl_3$): δ 2.03-2.09 (m, 1H, CH), 2.31-2.40 (m, 2H, CH_2), 2.49-2.57 (m, 1H, CH), 3.21-3.30 (m, 1H, CH), 3.80 (dd, J = 15.5, 36.0 Hz, 1H, CH), 4.22 (dd, J = 4.0, 8.0 Hz, 1H, CH), 4.45-4.58 (m, 2H, CH_2), 4.61 (d, J = 6.0 Hz, 2H, CH_2Ph), 6.53 (br s, 1H, CONH), 7.37 (t, J = 8.0 Hz, 1H, Ph-H), 7.59 (d, J = 8.0 Hz, 1H, Ph-H), 7.66 (d, J = 8.0 Hz, 1H, Ph-H). ^{13}C NMR ($CDCl_3$): δ 23.96, 29.51, 41.92, 42.67 (d, $J_{C\text{-}F}$ = 18.75 Hz), 62.55 (d, $J_{C\text{-}F}$ = 1.25 Hz), 82.36 (d, $J_{C\text{-}F}$ = 166.25 Hz, $\underline{C}H_2F$), 119.66 (q, $J_{C\text{-}F}$ = 271.25 Hz, $\underline{C}F_3$), 127.11, 127.31 (q, $J_{C\text{-}F}$ = 5.0 Hz), 129.05 (q, $J_{C\text{-}F}$ = 30.0 Hz), 131.85, 133.92, 137.73, 171.51, 176.21. MS (ESI): 367 ([M+H]$^+$, 100%); MS (ESI): 365 ([M-H]$^-$, 50%). HRMS (ESI): calcd for $C_{15}H_{16}N_2O_2ClF_4$ ([M+H]$^+$) 367.0836, found 367.0848.

*(g). (S)-N-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-chloroethyl)-5-oxopyrrolidine-2-carboxamide (Cl-IUR-1601, **3b**):* Compound **3b** was prepared using the same procedure as described for the synthesis of **3a** by substituting **2b** for **2a**. White solid, yield 27%, R_f = 0.30 (1:15 MeOH/CH_2Cl_2), mp 127-129 °C. ^{1}H NMR ($CDCl_3$): δ 2.03-2.06 (m, 1H, CH), 2.37-2.41 (m, 2H, CH_2), 2.48-2.52 (m, 1H, CH), 3.7-3.23 (m, 1H, CH), 3.58-3.63 (m, 1H, CH), 3.66-3.71 (m, 1H, CH), 3.94 (td, J = 5.0, 14.5 Hz, 1H, CH), 4.29 (dd, J = 3.5, 8.5Hz, 1H, CH), 4.62 (d, 2H, CH_2Ph), 6.44 (brs, 1H, CONH), 7.38 (t, J = 8.0 Hz, 1H, Ph-H), 7.60 (d, J = 8.0 Hz, 1H, Ph-H), 7.67 (dd, J = 1.0 , 8.0 Hz, 1H, Ph-H). MS (ESI): 383 ([M+H]$^+$, 100%); MS (ESI): 381 ([M-H]$^-$, 100%).

*(h). (S)-N-(2-Chloro-3-(trifluoromethyl)benzyl)-1-(2-[^{18}F]fluoroethyl)-5-oxopyrrolidine-2-carboxamide ([^{18}F]IUR-1601, [^{18}F]**3a**):* No-carrier-added (NCA) aqueous H[^{18}F]F was produced by ^{18}O(p,n)^{18}F nuclear reaction using a Siemens Eclipse RDS-111 cyclotron by irradiation of $H_2{}^{18}O$ (2.5 mL). H[^{18}F]F (~37 GBq) in [^{18}O]water plus 0.1 mL K_2CO_3 solution (1.7 mg) and Kryptofix 2.2.2 (10 mg) in 1.0 mL CH_3CN with additional 1 mL CH_3CN were placed in the fluorination reaction vial (5-mL V-vial) and repeated azeotropic distillation (17 min) was performed at 110 °C to remove water and to form the anhydrous K[^{18}F]F-Kryptofix 2.2.2 complex. $TsOCH_2CH_2OTs$ (14.8 mg) dissolved in CH_3CN (1.0 mL) was introduced to the reaction vessel and heated at 100-110 °C for 20 min to affect radiofluorination. After cooling to 80 °C, the reaction mixture 2-[^{18}F]FCH_2CH_2OTs was diluted with 3.0 mL H_2O, passed through a C18 Plus Sep-Pak cartridge, washed with H_2O (2 mL ×3), eluted with CH_3CN (2 mL × 2), transferred to a 10-mL V-vial, and repeated azeotropic distillation (17 min) was performed at 110 °C to remove water and to form the anhydrous 2-[^{18}F]FCH_2CH_2OTs. The precursor desmethyl-GSK1482160 (6 mg) and 2 M KOH (10 μL) in CH_3CN (1.0 mL) was introduced to the reaction vessel. The mixture was heated to 100-110 °C for 20 min, cooled to 80 °C, and then quenched with H_2O (2.0 mL). The mixture was injected onto the semi-preparative HPLC column with 3 mL injection loop for purification, using a YMC-Pack ODS-A, S-5 μm, 12 nm, 10×250 mm C18 (reverse phase) column; 40% CH_3CN:60% 3 mM $HCOONH_4$ mobile phase; 5.0 mL/min flow rate; UV (270 nm) and γ-ray (PIN diode) flow detectors. The product fraction was collected in a recovery vial containing 30 mL water. The diluted tracer solution was then passed through a C-18 Plus Sep-Pak cartridge, and washed with water (5 mL × 5). The cartridge was eluted with EtOH (1 mL × 2) to release [^{18}F]IUR-1601, followed by saline (10 mL). The eluted product was then sterile-filtered through a Millex-FG 0.2 μm membrane into a sterile vial. Total radioactivity was assayed and total volume was noted for tracer dose dispensing. Retention times in the semi-preparative HPLC system were: t_R desmethyl-GSK1482160 = 8.68 min, t_R vinyl-IUR-1601 = 11.35 min, t_R IUR-1601 = 13.37 min, t_R [^{18}F]IUR-1601 = 13.45 min, t_R 2-FCH_2CH_2OTs = 18.16 min, t_R 2-[^{18}F]FCH_2CH_2OTs = 18.29 min, and t_R $TsOCH_2CH_2OTs$ = 20.09 min. Retention times in the analytical HPLC system were: t_R IUR-1601 = 4.42 min and t_R [^{18}F]IUR-1601 = 4.59 min. The decay corrected radiochemical yields of [^{18}F]IUR-1601 were 1-3%.

(i). Cell culture and membrane preparation: HEK293 cells expressing human recombinant P2X7 receptor (hP2X7R) were obtained from B'SYS GmbH and cultured according to the supplier's procedure. Cells were grown to 80% confluency and then rinsed with phosphate-buffered saline (PBS), detached with trypsin, and harvested. Cell pellets were obtained by centrifugation at 200 *g* for 5 min at 4 °C. Collected cell pellets were frozen at −80 °C until membrane preparation. For the membrane preparation, pellets from 10 T225 flasks were homogenized in 50 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid (EDTA), and 140 mM NaCl at pH 7.4 and 4 °C. The homogenate was then centrifuged at 48,000 *g* for 20 min at 4 °C, gently rinsed with deionized water, and then resuspended in 50 mM Tris-HCl at pH 7.4 and 4 °C. This homogenate was then centrifuged as before,

with the resulting pellet being homogenized in 50 mM Tris-HCl at pH 7.4. Total protein concentration was determined via the Bradford protein assay (Bio-Rad). Aliquots were stored in cryovials at −80 °C until the day of assay.

(j). [^{11}C]GSK1482160 standards: For each experiment, the highest concentration of radioactivity used was diluted twice in assay buffer, followed by eleven 2-fold dilutions. Twenty microliters of each dilution were then spotted onto a GF/B UniFilter-96 plate (Perkin-Elmer) and allowed to air-dry until the end of the experiment. An additional 20 μL of each dilution were added to a scintillation vial containing 7 mL of Optiphase Hisafe 3 (Perkin-Elmer) and counted on an LS6000 scintillation counter (Beckman). Aliquots of the working concentrations of radioactivity used on each day were counted in the same manner.

(k). Radioligand competitive binding assay: For competitive binding assays, cell membrane preparation (0.054 mg of protein/mL of assay medium) was incubated with 11 compound concentrations over a six log unit range, with 5 nM [^{11}C]GSK1482160 in assay buffer (50 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumen (BSA)) . Triplicate determinations were done at each concentration of test compound. GSK1482160 was used to determine non-specific binding. Assays were incubated at 22 °C for 30 min. For termination of the binding reaction, the samples were filtered onto GF/B UniFilter plates that had been presoaked in 0.5% polyethyleneimine for 30 min using a UniFilter-96 cell harvester. The plates were washed 5 times with ice-cold saline, dried under a vacuum, and exposed to a TR2025 phosphor screen for 20–60 min. Phosphor screens were then read on a Typhoon FLA-7000IP (GE Healthcare) along with [^{11}C]-calibration standards. CPMs (counts per minute) were determined by calibrating the image to the CPMs in the calibration standards via MCID analysis Software. Data was analyzed with Prism 7 (GraphPad Software Inc.) to determine K_i values.