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Short communication

Ether analogues of DPA-714 with subnanomolar affinity for the translocator protein (TSPO)

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ABSTRACT

Sixteen new phenyl alkyl ether derivatives (**12**, **14**–**28**) of the 5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-ylacetamide (DPA) class were synthesized and evaluated in a competition binding assay against [^{3}H]PK11195 using 18 kDa translocator protein (TSPO) derived from rat kidney mitochondrial fractions. All analogues showed superior binding affinities for TSPO compared to DPA-713 (**5**) and DPA-714 (**6**). Picomolar affinities were observed for this class of TSPO ligands in this assay for the first time, with phenethyl ether **28** showing the greatest affinity (K_i = 0.13 nM). Additionally, all analogues increased pregnenolone biosynthesis (134–331% above baseline) in a rat C6 glioma cell steroidogenesis assay.

1. Introduction

The 18 kDa translocator protein (TSPO) is a five transmembrane domain protein subunit of a multimeric complex located primarily in the outer mitochondrial membrane, and is mainly responsible for the translocation of cholesterol from the outer-to inner-mitochondrial membrane [1,2]. TSPO is widely expressed in peripheral organs, especially in steroidogenic tissues. Normal TSPO expression in the brain is minimal [3], but several neuropathologies (including stroke, Alzheimer's disease, and Parkinson's disease) are associated with an overexpression of TSPO in the brain [4]. TSPO is currently thought to be a biomarker for microglial activation and neuroinflammation [5], as well as a potential therapeutic target for neurological and psychiatric disorders [4,6–8].

Beyond its role in cholesterol metabolism and steroid biosynthesis, TSPO is also involved in cell proliferation and apoptosis [8]. The overexpression of TSPO has been observed in numerous human cancers, particularly breast cancer [9–11]. For breast cancers, TSPO expression changes with cancer progression, and is associated with aggressive phenotypes [12]. In the case of neuroblastoma, TSPO ligands were able to induce apoptosis and cell cycle arrest, and offer sensitization to chemotherapy [13]. For these reasons, TSPO has been promoted as a novel target for cancer imaging as well as cancer therapies [14,15].

One of the earliest and most widely explored TSPO ligands is the quinoline carboxamide PK11195 (**1**, Fig. 1). The affinity of PK11195 for TSPO is in the nanomolar range in both rat (K_i = 3.0 nM) [16] and human (K_i = 4.5–22.3 nM) [17,18]. Another structural class of high affinity TSPO ligands are the substituted acetanilides typified by DAA1106 (**2**) [19]. DAA1106 demonstrates picomolar affinity for rat TSPO (K_i = 0.0726 nM) [17], but obvious species differences exist for this compound, and reduced affinities are observed for human TSPO (K_i = 0.2–13.1 nM) [17,20]. The structurally-related acetamide PBR28 (**3**) shows a TSPO species difference comparable to **2**, with greater affinity for rat TSPO (K_i = 0.7 nM) than human TSPO (K_i = 2.5–4.0 nM) [17,18,20]. More recently, tetrahydrocarbazole

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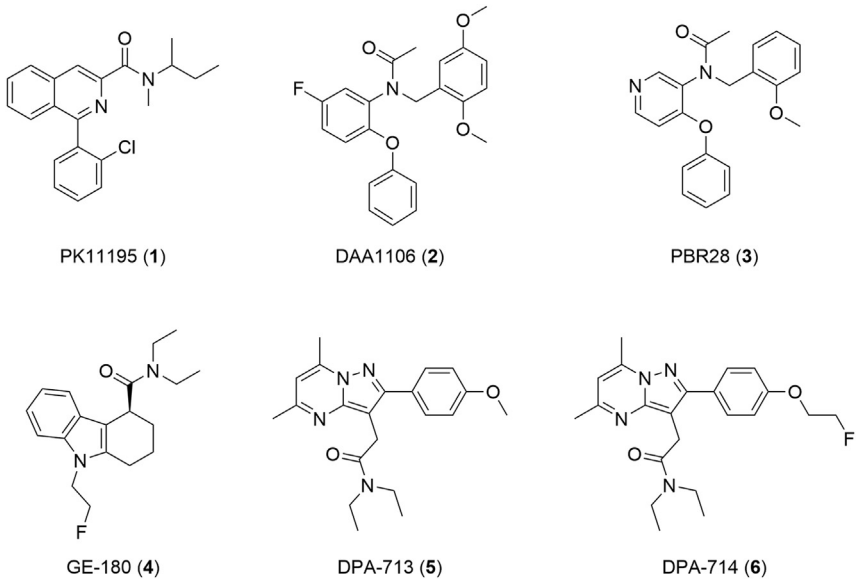


Fig. 1. Selected high affinity TSPO ligands.

GE-180 (**4**, K_i = 0.87 nM) was identified as part of a program to reduce the lipophilicity of a tetracyclic lead structure [21], and [^{18}F]GE-180 is currently under investigation as a TSPO PET imaging agent.

The 5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-ylacetamide (DPA) scaffold has been used to generate selective, drug-like ligands targeting TSPO. The most well-characterized examples of this class are DPA-713 (**5**, K_i = 4.7 nM vs. [^{3}H]**1** in TSPO derived from rat kidney) and DPA-714 (**6**, K_i = 7.0 nM vs. [^{3}H]**1** in TSPO derived from rat kidney) [22–24]. In a quinolinic acid model of excitotoxic injury in rat, **5** and **6** inhibited microglial activation and increased neuronal survival, indicating neuroprotective effects [25]. Additionally, [^{11}C]**5** and [^{18}F]**6** have demonstrated utility as radiotracers for PET imaging of TSPO in humans, highlighting the *in vivo* suitability of the DPA class for diagnostic and therapeutic drug development [26–31].

Previous structure–activity relationship (SAR) studies of the DPA class of TSPO ligands have focused on the generation of analogue libraries containing variation of the pyrazolopyrimidine core and amide group, with limited exploration of the phenyl alkyl ether region [22,32,33]. The present study systematically explored the steric and electronic tolerance of the TSPO binding site for the ether region of DPA-713 and DPA-714, with the aim of utilizing the obtained SAR information to guide the development of increasingly potent analogues of DPA-713 and DPA-714 for therapeutic investigation, as well as high affinity structures amenable to the incorporation of C-11 and F-18 radioisotopes as PET imaging candidates. To this end, analogues of DPA-713 and DPA-714 containing aliphatic, alicyclic, and aromatic groups as pendant substituents, at various distances from the ether linkage, were prepared.

2. Synthetic chemistry

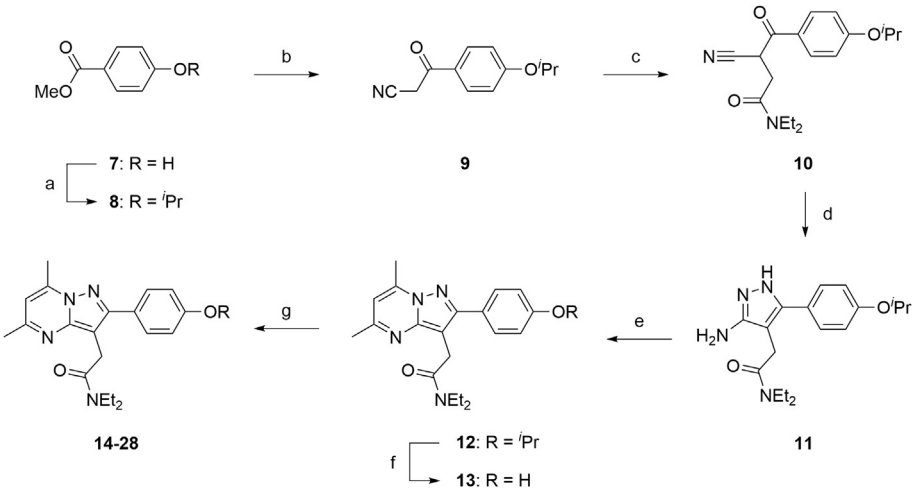
The synthesis of novel DPA analogues (Scheme 1) is based on a scalable general method recently developed in our laboratories [34]. Commercial methyl 4-hydroxybenzoate (**7**) was quantitatively protected as its isopropyl ether (**8**), and treated with the conjugate base of acetonitrile to generate cyanoacetophenone **9**. Deprotonation of **9** to the corresponding enolate, followed by treatment with excess *N*,*N*-diethylbromoacetamide, gave crude **10**, which was immediately condensed with hydrazine to yield aminopyrazole **11**. Condensation of **11** with acetylacetone generated the pyrazolopyrimidine core of **12**, and subsequent cleavage of the isopropyl ether using aluminum chloride afforded common phenolic precursor **13**. Alkylation of **13** was achieved by mild heating in the presence of the appropriate alkyl bromide and potassium carbonate, to cleanly furnish DPA analogues **14**–**28** in 37–90% yield following purification by flash chromatography or recrystallization.

3. Results and discussion

The synthesized DPA analogues **14**–**28** and PK11195 were evaluated for TSPO affinity using a membrane binding assay with [^{3}H]PK11195 as the competitive radioligand, and mitochondrial fractions from rat kidney as the TSPO source. The results are summarized in Table 1. The selectivity of DPA-713, DPA-714, and several simple *n*-alkyl analogues for TSPO was previously demonstrated by examining binding to the central benzodiazepine receptor (CBR) using [^{3}H]Ro15,1788 and rat brain tissue [24]. Several simple alkyl ether analogues from the DPA structural class of TSPO ligands were shown to possess selectivity over CBR as well as numerous other common CNS targets [24]. Off-target activity of these novel DPA analogues was not determined since the aim of the present study was to explore SARs specifically for the ether region of the DPA class of TSPO ligands.

The DPA analogues containing simple branched alkyl ethers, such as an isopropyl (**12**, K_i = 2.2 nM) or isobutyl (**14**, K_i = 2.4 nM) group, showed similar affinities to DPA-713 and DPA-714 (K_i = 4.7 nM and 7.0 nM, respectively), suggesting some steric tolerance at the TSPO binding site. TSPO binding was improved by the introduction of alicyclic rings, with a cyclobutyl (**15**, K_i = 0.86 nM) or cyclopentyl (**16**, K_i = 1.5 nM) ether both conferring low- or subnanomolar affinities. The synthesis of cyclopropyl and cyclohexyl analogues from bromocyclopropane and bromocyclohexane was attempted, however, these alkylations proceeded slowly and only traces of expected ether compound could be obtained after several days of reaction at 40 °C. Attempts to increase reaction rate by elevating temperature resulted in the formation of significant quantities of unidentified by-products.

Spacing the alicyclic group from the phenyl ether by a single



Scheme 1. Reagents and conditions: (a) ^{i}PrBr (1.5 eq.), K_2CO_3 (3.5 eq.), Me_2CO, reflux, 72 h, 100%; (b) NaH (2 eq.), MeCN (5 eq.), PhMe, 80 °C, 18 h, 67%; (c) NaH (2.5 eq.), $BrCH_2C(O)NEt_2$, THF, 0 °C–rt, 27 h; (d) $NH_2NH_2{\cdot}H_2O$ (2 eq.), AcOH (1.6 eq.), EtOH, reflux, 13 h, 71% over 2 steps; (e) $CH_3C(O)CH_2C(O)CH_3$, EtOH, reflux, 20 h, 91%; (f) $AlCl_3$ (3.3 eq.), CH_2Cl_2, 0 °C–rt, 16 h, 86%; (g) RBr (1.5 eq.), K_2CO_3 (5 eq.), DMF, 40–50 °C, 14–90 h, 37–90%.

methylene unit gave a homologous alicyclic series featuring a cyclopropyl (**17**), -butyl (**18**), -pentyl (**19**), or -hexyl ring (**20**). As with the alkyl congeners, TSPO binding affinities for this series were in the low nanomolar range (K_i = 1.4–3.6 nM), further confirming steric tolerance at the complementary TSPO binding site.

Given the nanomolar affinity of **20**, it was hypothesized that benzylic ethers might also furnish high affinity TSPO ligands. Additionally, such compounds offer the potential for physicochemical "fine-tuning" by judicious incorporation of substituents around the ring, as well as the incorporation of pendant carbon-11 and fluorine-18 isotopes for PET imaging. The simplest benzylic DPA analogue, **21** (K_i = 0.99 nM), demonstrated comparable binding affinity to the alkyl and alicyclic derivatives. Systematic introduction of a fluorine atom to **21** to give all possible fluorobenzyl regioisomers resulted in picomolar TSPO binding in each case. A 2-fluoro substituent (**22**, K_i = 0.31 nM) produced the greatest TSPO affinity, followed by the 3- (**23**, K_i = 0.47 nM) and 4-positions (**24**, K_i = 0.77 nM). Similar regioisomeric incorporation of the more electronegative and sterically demanding trifluoromethyl group was less effective. Although only a 3-trifluoromethyl group (**26**, K_i = 0.67 nM) conferred subnanomolar binding, analogues containing a trifluoromethyl group in the 2- (**25**, K_i = 1.4 nM) or 4-position (**27**, K_i = 1.6 nM) retained low nanomolar affinities. Further homologation of **21** produced the most high affinity pyrazolopyrimidine TSPO ligand thus identified, phenethyl DPA derivative **28** (K_i = 0.13 nM).

All new DPA-714 analogues were evaluated for their ability to increase pregnenolone biosynthesis in rat C6 glioma cells using a well-established steroidogenesis assay [22,35–37]. Compounds **12**, **14**–**28**, and PK11195 were tested at the same concentration (40 μM) to allow direct comparison of steroidogenic efficacy. After a two hour incubation period, pregnenolone was quantified using an immunoenzymatic assay. The results are shown in Fig. 2, and a complete table of the data is available in the SI.

All new DPA analogues, as well as PK11195, increased pregnenolone production. Rates of pregnenolone biosynthesis increased from 134 to 331% of that induced by drug vehicle (DMSO) alone. Of the aliphatic and alicyclic DPA-714 analogues, cyclopentyl ether **16** and methylene-spaced cyclobutyl derivative **18** showed the highest efficacy (273% and 298% respectively). Isobutyl ether **14** and methylene-spaced cyclopropyl derivative **17** have similar lipophilicities and steric demands and demonstrated similar efficacy in this assay (206% and 226% respectively). All other aliphatic and alicyclic ether analogues were approximately as efficacious as PK11195 (166%). Excluding **22** and **27**, all benzylic and phenethyl DPA-714 analogues demonstrated greater than 230% increase in pregnenolone biosynthesis, with (2-trifluoromethyl)benzyl **25** proving the most potent (331%).

A correlation between binding affinity and steroidogenic efficacy was established and is plotted in Fig. 3. The relationship was significant when all new ligands (**12** and **14**–**28**) were included, however, correlation was improved by exclusion of **22** as an outlier (details of each analysis are available in Figs. S2 and S3 of the SI). Despite having the second highest binding affinity of the series (K_i = 0.31 nM), **22** was also the second-least efficacious DPA-714 analogue in the pregnenolone biosynthesis assay (162%) after **20** (134%).

These results suggest that binding affinity is linked to steroidogenic activity for ether analogues of DPA-714 possessing similar binding modes.

4. Conclusion

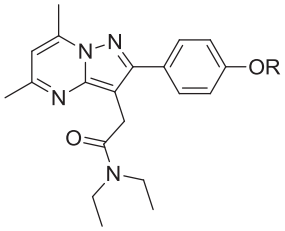
All novel DPA analogues showed higher affinity for TSPO than DPA-713 and DPA-714, with **28** showing the greatest improvement, and **20** showing the least. The improvements to TSPO binding for the series ranged from 1.3 to 36 times compared to DPA-713, and 1.9 to 53 times for DPA-714. The high affinities of benzylic congeners **22**–**27** indicate that substituents of varying electron density are tolerated at all positions around the ring. The low- or subnanomolar affinities of **12** and **14**–**28** collectively indicate broad steric and electronic tolerance by the TSPO binding site for the phenyl alkyl ether region of DPA-713 and DPA-714. In particular, the high affinities of **21**–**28**, and potent steroidogenic activities of **21**, **23**–**26**, and **28**, suggest that ring-substituted benzylic analogues of DPA-714 may offer new directions for the development of novel, high affinity TSPO ligands with potential therapeutic applications.

5. Experimental procedures

5.1. General chemistry details

All reactions were performed under an atmosphere of nitrogen or argon unless otherwise specified. Dichloromethane was distilled

Table 1
TSPO affinities of DPA analogues **1**, **5**, **6**, **12**, and **14**–**28**.



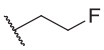
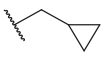
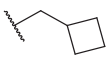
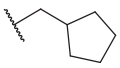
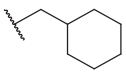
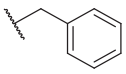
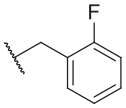
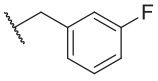
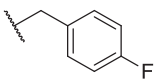
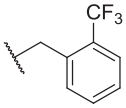
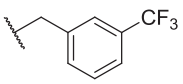
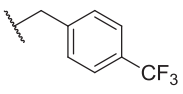
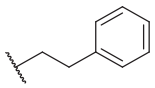
Compound	R	TSPO K_i (nM ± SEM)[a]
1 (PK11195)	–	9.3 ± 0.5[b]
5 (DPA-713)		4.7 ± 0.2[b]
6 (DPA-714)		7.0 ± 0.4[b]
12		2.2 ± 0.2
14		2.4 ± 0.3
15		0.86 ± 0.08
16		1.5 ± 0.2
17		3.0 ± 0.3
18		1.4 ± 0.2
19		2.1 ± 0.2
20		3.6 ± 0.5
21		0.99 ± 0.09
22		0.31 ± 0.05
23		0.47 ± 0.045
24		0.77 ± 0.08
25		1.4 ± 0.2
26		0.67 ± 0.07
27		1.6 ± 0.2

Table 1 (*continued*)

Compound	R	TSPO K_i (nM ± SEM)[a]
28		0.13 ± 0.01

[a] K_i values represent the mean ± SEM of three experiments.
[b] Data extracted from Ref. [24].

from calcium hydride. Commercially available chemicals were used as purchased. Analytical thin layer chromatography (TLC) was performed using Merck aluminum-backed silica gel 60 F254 (0.2 mm) plates which were visualized using shortwave (254 nm) and/or longwave (365 nm) ultra-violet fluorescence. Flash chromatography was performed using Merck Kieselgel 60 (230–400 mesh) silica gel. Melting points were measured in open capillaries using a Stuart SMP10 Melting Point Apparatus and are uncorrected. Infrared absorption spectra were recorded on a Bruker ALPHA FT-IR spectrometer, and the data are reported as vibrational frequency (cm^{-1}). Nuclear magnetic resonance spectra were recorded at 300 K using either a Bruker AVANCE DRX400 (400.1 MHz) or AVANCE III 500 Ascend (500.1 MHz) spectrometer. The data is reported as chemical shift (δ ppm) relative to the residual protonated solvent resonance, relative integral, multiplicity (s = singlet, br s = broad singlet, d = doublet, t = triplet, q = quartet, sep = septet, m = multiplet), coupling constants (J Hz), and assignment. Assignment of signals was assisted by COSY, DEPT, HSQC, and HMBC experiments where necessary. Low resolution mass spectra (LRMS) were recorded using electrospray ionization (ESI) recorded on a Finnigan LCQ ion trap spectrometer. Elemental analysis was obtained from the Chemical Analysis Facility in the Department of Chemistry and Biomolecular Sciences, Macquarie University, Australia.

5.1.1. 2-(2-(4-Isopropoxyphenyl)-5,7-dimethylpyrazolo[1,5-a] pyrimidin-3-yl)-N,N-diethylacetamide (12)

Compound **12** was synthesized from methyl 4-hydroxybenzoate (**7**) in 5 steps and 43% overall yield using a previously described procedure [34]. m.p. 163–164 °C; R_f 0.53 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74 (2H, d, J = 9.0 Hz, ArH), 6.96 (2H, d, J = 9.0 Hz, ArH), 6.49 (1H, s, ArH), 4.59 (1H, sep, J = 6.1 Hz, OCH), 3.90 (2H, s, $ArCH_2$), 3.49 (2H, q, J = 7.0 Hz, NCH_2), 3.40 (2H, q, J = 7.0 Hz, NCH_2), 2.73 (3H, s, $ArCH_3$), 2.53 (3H, s, $ArCH_3$), 1.35 (6H, d, J = 6.1 Hz, $OCHCH_3$), 1.19 (3H, t, J = 7.0 Hz, CH_2CH_3), 1.12 (3H, t, J = 7.0 Hz, CH_2CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.3 (C=O), 158.3 ($C_{quat.}$), 157.6 ($C_{quat.}$), 154.3 ($C_{quat.}$), 147.8 ($C_{quat.}$), 144.8 ($C_{quat.}$), 130.1 (2C, CH), 126.2 ($C_{quat.}$), 116.1 (2C, CH), 108.2 (CH), 100.9 ($C_{quat.}$), 70.0 (OCH), 42.4 (NCH_2), 40.7 (NCH_2), 28.3 (CH_2), 24.8 (CH_3), 22.2 (2C, $OCHCH_3$), 17.1 (CH_3), 14.5 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 395.67 ($[M+H]^+$, 100%); Anal. ($C_{23}H_{30}N_4O_2$): calcd, C 70.02, H 7.66, N 14.20; found, C 71.04, H 7.82, N 14.52.

5.1.2. 2-(2-(4-Hydroxyphenyl)-5,7-dimethylpyrazolo[1,5-a] pyrimidin-3-yl)-N,N-diethylacetamide (13)

A cooled (0 °C) solution of **12** (1.58 g, 4.0 mmol) in dichloromethane (12.5 mL) was treated portionwise with aluminum chloride (1.76 g, 13.0 mmol, 3.3 equiv.) and the solution allowed to warm to rt. After several minutes, a heterogeneous mixture formed and was stirred vigorously for 20 h. The reaction mixture was cooled (0 °C), treated with half sat. aq. NH_4Cl (40 mL), stirred for 0.5 h, then allowed to warm to rt and stirred a further 2 h. The mixture was filtered and the precipitate washed sequentially with H_2O (40 mL), $CHCl_3$ (40 mL), and set aside. The filtrate layers were separated and the aqueous phase was extracted with $CHCl_3$

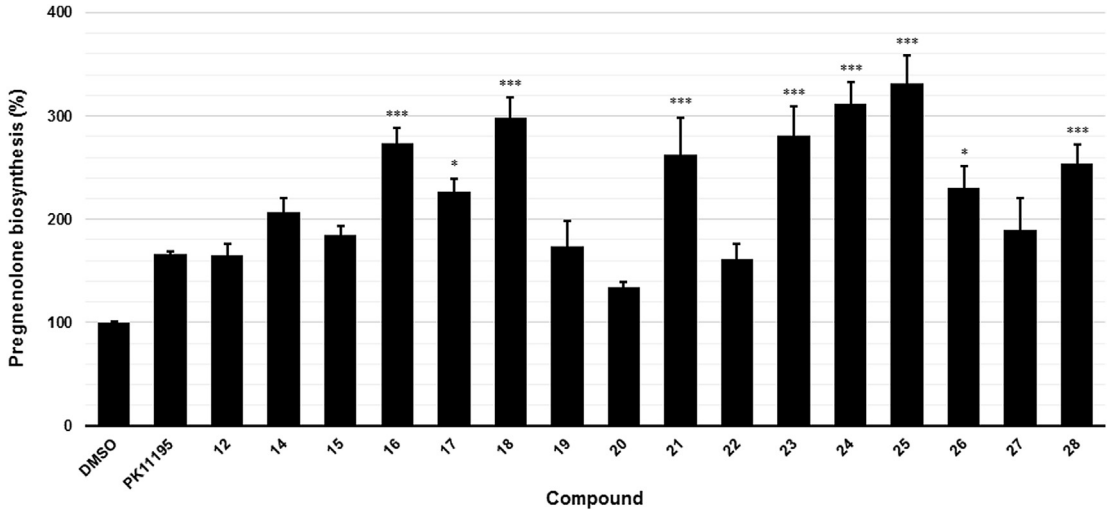


Fig. 2. Pregnenolone biosynthesis in rat C6 glioma cells induced by selected TSPO ligands. (Each value represents the mean ± S.E.M. of 6–9 replicates; $^{*}P < 0.05$, $^{***}P < 0.001$).

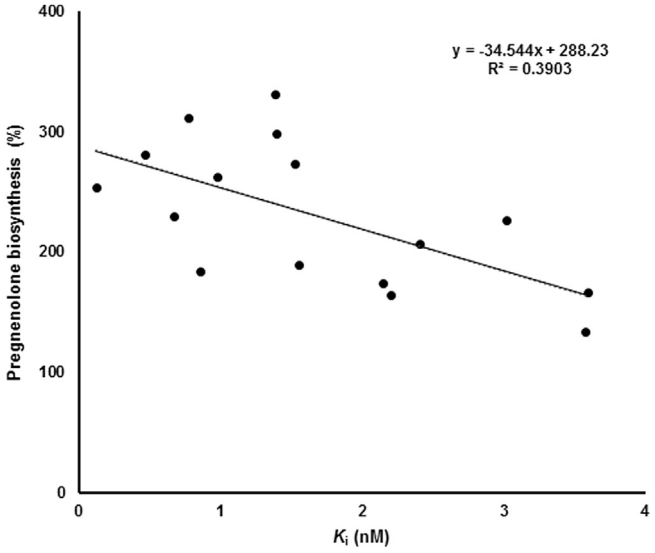


Fig. 3. Correlation between binding affinity and steroidogenic activity for **12**, **14**–**19**, and **21**–**28**.

(2 × 15 mL). The combined organic phases were washed with H_2O (30 mL), brine H_2O (30 mL), dried ($MgSO_4$), and the solvent evaporated. The residue thus obtained was combined with the precipitate previously set aside, dissolved in $CHCl_3$–MeOH (50:50) and dry-loaded on silica gel, and eluted through a short plug of silica using $CHCl_3$–MeOH (90:10) as eluent. The solvent was evaporated under reduced pressure and the remaining solid triturated with Et_2O to give **8** (1.21 g, 86%) as a pale yellow crystalline solid of suitable purity for use in the following alkylation reactions (*vide infra*). Analytical purity was achieved by recrystallization from isopropanol to give colorless crystals. m.p. 247–249 °C; R_f 0.12 ($CHCl_3$–MeOH, 95:5); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.64 (2H, d, J = 8.5 Hz, ArH), 7.13 (1H, br s, ArOH), 6.82 (2H, d, J = 8.5 Hz, ArH), 6.49 (1H, s, ArH), 3.95 (2H, s, $ArCH_2$), 3.50 (2H, q, J = 7.2 Hz, NCH_2), 3.38 (2H, q, J = 7.2 Hz, NCH_2), 2.73 (3H, s, $ArCH_3$), 2.54 (3H, s, $ArCH_3$), 1.16 (3H, t, J = 7.2 Hz, $CH_2\underline{CH_3}$), 1.09 (3H, t, J = 7.2 Hz, $CH_2\underline{CH_3}$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.7 (C=O), 157.7 ($C_{quat.}$), 157.0 ($C_{quat.}$), 155.6 ($C_{quat.}$), 147.7 ($C_{quat.}$), 145.0 ($C_{quat.}$), 130.2 (2C, CH), 125.5 ($C_{quat.}$), 115.8 (2C, CH), 108.3 (CH), 100.6 ($C_{quat.}$), 42.7 (NCH_2), 41.0 (NCH_2), 28.5 ($ArCH_2$), 24.7 ($ArCH_3$), 17.1 ($ArCH_3$), 14.3 ($CH_2\underline{CH_3}$), 13.1 ($CH_2\underline{CH_3}$); LRMS (+ESI) m/z 353.53 ($[M+H]^+$, 100%); Anal. ($C_{20}H_{24}N_4O_2$): calcd, C 68.16, H 6.86, N 15.90; found, C 68.33, H 7.23, N 15.72. All physical and spectroscopic data matched those previously reported [24].

5.2. General procedure for the alkylation of 13

A mixture of **13** (176 mg, 0.5 mmol, 1 equiv.), anhydrous K_2CO_3 powder (346 mg, 2.5 mmol, 5 equiv.), and the appropriate bromoalkane (0.75 mmol, 1.5 equiv.) in DMF (1 mL) was stirred 40 °C for 14 h. The mixture was poured into H_2O (50 mL), and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (10 mL), dried ($MgSO_4$), and the solvent evaporated. The crude products were purified by flash chromatography eluting with $CHCl_3$–MeOH (96:4) or recrystallized from isopropanol.

5.2.1. 2-(2-(4-Isobutoxyphenyl)-5,7-dimethylpyrazolo[1,5-a] pyrimidin-3-yl)-N,N-diethylacetamide (14)

Treating **13** with 1-bromo-2-methylpropane (80 μL, 0.75 mmol, 1.5 equiv.) according to the general procedure gave **14** as a colorless crystalline solid (175 mg, 86%). m.p. 116–118 °C; R_f 0.44 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 (2H, d, J = 8.5 Hz, ArH), 6.96 (2H, d, J = 8.5 Hz, ArH), 6.47 (1H, s, ArH), 3.90 (2H, s, $ArCH_2$), 3.75 (2H, d, J = 6.5 Hz, OCH_2), 3.49 (2H, q, J = 7.0 Hz, NCH_2), 3.40 (2H, q, J = 7.0 Hz, NCH_2), 2.71 (3H, s, $ArCH_3$), 2.51 (3H, s, $ArCH_3$), 2.09 (1H, app. sep., J = 6.5 Hz, $C\underline{H}$), 1.19 (3H, t, J = 7.0 Hz, $CH_2\underline{CH_3}$), 1.10 (3H, t, J = 7.0 Hz, $CH_2\underline{CH_3}$), 1.03 (6H, d, J = 6.5 Hz, $CH(\underline{CH_3})_2$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2 (C=O), 159.6 ($C_{quat.}$), 157.4 ($C_{quat.}$), 155.2 ($C_{quat.}$), 147.7 ($C_{quat.}$), 144.8 ($C_{quat.}$), 130.0 (2C, CH), 126.2 ($C_{quat.}$), 114.7 (2C, CH), 108.1 ($C_{quat.}$), 100.8 ($C_{quat.}$), 74.5 (OCH_2), 42.4 (NCH_2), 40.7 (NCH_2), 28.35 (CH_2), 28.28 (CH), 24.7 (CH_3), 19.3 (2C, $CH(\underline{CH_3})_2$), 17.0 (CH_3), 14.4 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 409.07 ($[M+H]^+$, 100%); Anal. ($C_{24}H_{32}N_4O_2$): calcd, C 70.56, H 7.90, N 13.71; found, C 70.55, H 8.10, N 13.71.

5.2.2. 2-(2-(4-Cyclobutoxyphenyl)-5,7-dimethylpyrazolo[1,5-a] pyrimidin-3-yl)-N,N-diethylacetamide (15)

Treating **13** with bromocyclobutane (70 μL, 0.75 mmol, 1.5 equiv.) for 5 days according to the general procedure gave **15** as a colorless crystalline solid (135 mg, 66%, 82% brsm). m.p. 157–159 °C; R_f 0.54 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.72 (2H, d, J = 9.0 Hz, ArH), 6.89 (2H, d, J = 9.0 Hz, ArH),

6.52 (1H, s, ArH), 4.69 (1H, quin., J = 7.3 Hz, OCH), 3.99 (2H, s, $ArCH_2$), 3.51 (2H, q, J = 7.3 Hz, NCH_2), 3.41 (2H, q, J = 7.3 Hz, NCH_2), 2.77 (3H, s, $ArCH_3$), 2.60 (3H, s, $ArCH_3$), 2.50–2.44 (2H, m, $CHCH_2$), 2.23–2.15 (2H, m, $CHCH_2$), 1.91–1.84 (1H, m, $CHCH_2CH_2$), 1.75–1.67 (1H, m, $CHCH_2CH_2$), 1.22 (3H, t, J = 7.3 Hz, CH_2CH_3), 1.12 (3H, t, J = 7.3 Hz, CH_2CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.0 (C=O), 157.9 ($C_{quat.}$), 157.5 ($C_{quat.}$), 155.3 ($C_{quat.}$), 147.8 ($C_{quat.}$), 144.8 ($C_{quat.}$), 130.1 (2C, CH), 126.4 ($C_{quat.}$), 115.2 (2C, CH), 108.2 (CH), 100.9 ($C_{quat.}$), 71.6 (OCH), 42.4 (NCH_2), 40.7 (NCH_2), 30.8 (2C, CH_2), 28.3 (CH_2), 24.8 (CH_3), 17.0 (CH_3), 14.5 (CH_3), 13.4 (CH_2), 13.2 (CH_3); LRMS (+ESI) m/z 407.07 ($[M+H]^+$, 100%); Anal. ($C_{24}H_{30}N_4O_2$): calcd, C 70.91, H 7.44, N 13.78; found, C 70.95, H 7.54, N 13.85.

5.2.3. 2-(2-(4-Cyclopentyloxyphenyl)-5,7-dimethylpyrazolo[1,5-a] pyrimidin-3-yl)-N,N-diethylacetamide (16)

Treating **13** with bromocyclopentane (80 μL, 0.75 mmol, 1.5 equiv.) for 5 days at 45 °C according to the general procedure gave **16** as a colorless crystalline solid (198 mg, 47%). m.p. 130–132 °C; R_f 0.42 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.73 (2H, d, J = 9.0 Hz, ArH), 6.94 (2H, d, J = 9.0 Hz, ArH), 6.47 (1H, s, ArH), 4.80 (1H, sep., J = 2.9 Hz, OCH), 3.90 (2H, s, $ArCH_2$), 3.49 (2H, q, J = 7.2 Hz, NCH_2), 3.40 (2H, q, J = 7.2 Hz, NCH_2), 2.72 (3H, s, $ArCH_3$), 2.52 (3H, s, $ArCH_3$), 1.95–1.76 (6H, m), 1.65–1.57 (2H, m), 1.19 (3H, t, J = 7.2 Hz, CH_2CH_3), 1.10 (3H, t, J = 7.2 Hz, CH_2CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2 (C=O), 158.5 ($C_{quat.}$), 157.5 ($C_{quat.}$), 155.3 ($C_{quat.}$), 147.7 ($C_{quat.}$), 144.8 ($C_{quat.}$), 130.1 (2C, CH), 125.9 ($C_{quat.}$), 115.7 (2C, CH), 108.2 (CH), 100.8 ($C_{quat.}$), 79.3 (OCH), 42.4 (NCH_2), 40.7 (NCH_2), 32.9 (CH^2), 28.3 (CH_2), 24.7 (CH_3), 24.1 (2C, $OCHCH_3$), 17.0 (CH_3), 14.4 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 421.13 ($[M+H]^+$, 100%); Anal. ($C_{25}H_{32}N_4O_2$): calcd, C 71.40, H 7.67, N 13.32; found, C 71.49, H 7.87, N 13.23.

5.2.4. 2-(2-(4-Cyclopropylmethoxyphenyl)-5,7-dimethylpyrazolo [1,5-a]pyrimidin-3-yl)-N,N-diethylacetamide (17)

Treating **13** with (bromomethyl)cyclopropane (73 μL, 0.75 mmol, 1.5 equiv.) for 20 h according to the general procedure gave **17** as a colorless crystalline solid (176 mg, 86%). m.p. 122–124 °C; R_f 0.38 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 (2H, d, J = 9.0 Hz, ArH), 6.97 (2H, d, J = 9.0 Hz, ArH), 6.48 (1H, s, ArH), 3.90 (2H, s, $ArCH_2$), 3.84 (2H, d, J = 7.0 Hz, OCH_2), 3.49 (2H, q, J = 7.2 Hz, NCH_2), 3.40 (2H, q, J = 7.2 Hz, NCH_2), 2.73 (3H, s, $ArCH_3$), 2.53 (3H, s, $ArCH_3$), 1.30–1.27 (1H, m, OCH_2CH), 1.19 (3H, t, J = 7.2 Hz, CH_2CH_3), 1.11 (3H, t, J = 7.2 Hz, CH_2CH_3), 0.66–0.63 (2H, m, $CHCH_2$), 0.38–0.35 (2H, m, $CHCH_2$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2 (C=O), 159.4 ($C_{quat.}$), 157.5 ($C_{quat.}$), 155.2 ($C_{quat.}$), 147.8 ($C_{quat.}$), 144.8 ($C_{quat.}$), 130.1 (2C, CH), 126.4 ($C_{quat.}$), 114.8 (2C, CH), 108.2 (CH), 100.9 ($C_{quat.}$), 72.9 (OCH_2), 42.4 (NCH_2), 40.7 (NCH_2), 28.3 (CH_2), 24.7 (CH_3), 17.0 (CH_3), 14.5 (CH_3), 13.2 (CH_3), 10.4 (OCH_2CH), 3.31 (2C, $CHCH_2$); LRMS (+ESI) m/z 407.07 ($[M+H]^+$, 100%); Anal. ($C_{24}H_{30}N_4O_2$): calcd, C 70.91, H 7.44, N 13.78; found, C 70.98, H 7.62, N 13.80.

5.2.5. 2-(2-(4-Cyclobutylmethoxyphenyl)-5,7-dimethylpyrazolo [1,5-a]pyrimidin-3-yl)-N,N-diethylacetamide (18)

Treating **13** with (bromomethyl)cyclobutane (85 μL, 0.75 mmol, 1.5 equiv.) according to the general procedure gave **18** as a colorless crystalline solid (189 g, 90%). m.p. 107–109 °C; R_f 0.47 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 (2H, d, J = 9.0 Hz, ArH), 6.97 (2H, d, J = 9.0 Hz, ArH), 6.49 (1H, s, ArH), 3.97 (2H, d, J = 6.5 Hz, OCH_2), 3.91 (2H, s, $ArCH_2$), 3.49 (2H, q, J = 7.2 Hz, NCH_2), 3.41 (2H, q, J = 7.2 Hz, NCH_2), 2.80–2.77 (1H, m, OCH_2CH), 2.72 (3H, s, $ArCH_3$), 2.53 (3H, s, $ArCH_3$), 2.17–2.13 (2H, m, cyclobutane), 1.98–1.86 (2H, m, cyclobutane), 1.20 (3H, t, J = 7.1 Hz, CH_2CH_3), 1.11 (3H, t, J = 7.1 Hz, CH_2CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2 (C=O), 159.7 ($C_{quat.}$), 157.5 ($C_{quat.}$), 155.2 ($C_{quat.}$), 147.8 ($C_{quat.}$), 144.9 ($C_{quat.}$), 130.0 (2C, CH), 126.3 ($C_{quat.}$), 114.8 (2C, CH), 108.2 (CH), 100.9 ($C_{quat.}$), 72.3 (OCH_2), 42.4 (NCH_2), 40.7 (NCH_2), 34.8 (CH), 29.8 (CH_2), 28.3 (CH_2), 25.0 (2C, CH_2), 24.7 (CH_3), 18.7 (CH_2), 17.0 (CH_3), 14.5 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 421.13 ($[M+H]^+$, 100%); Anal. ($C_{25}H_{32}N_4O_2$): calcd, C 71.40, H 7.67, N 13.32; found, C 71.46, H 7.62, N 13.27.

5.2.6. 2-(2-(4-Cyclopentylmethoxyphenyl)-5,7-dimethylpyrazolo [1,5-a]pyrimidin-3-yl)-N,N-diethylacetamide (19)

Treating **13** with (iodomethyl)cyclopentane (100 μL, 0.75 mmol, 1.5 equiv.) at 50 °C for 5 days according to the general procedure gave **19** as a colorless crystalline solid (84 mg, 37%, 78% brsm). m.p. 107–108 °C; R_f 0.45 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74 (2H, d, J = 9.0 Hz, ArH), 6.97 (2H, d, J = 9.0 Hz, ArH), 6.49 (1H, s, ArH), 3.91 (2H, s, $ArCH_2$), 3.87 (2H, d, , J = 7.0 Hz, OCH_2), 3.49 (2H, q, J = 7.2 Hz, NCH_2), 3.41 (2H, q, J = 7.2 Hz, NCH_2), 2.73 (3H, s, $ArCH_3$), 2.53 (3H, s, $ArCH_3$), 2.42–2.33 (1H, m, OCH_2CH), 1.88–1.82 (2H, m), 1.68–1.56 (4H, m), 1.41–1.34 (2H, m), 1.20 (3H, t, J = 7.0 Hz, CH_2CH_3), 1.11 (3H, t, J = 7.0 Hz, CH_2CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.3 (C=O), 159.7 ($C_{quat.}$), 157.5 ($C_{quat.}$), 155.3 ($C_{quat.}$), 147.8 ($C_{quat.}$), 144.9 ($C_{quat.}$), 130.0 (2C, CH), 126.2 ($C_{quat.}$), 114.8 (2C, CH), 108.2 (CH), 100.9 ($C_{quat.}$), 72.5 (OCH_2), 42.4 (NCH_2), 40.7 (NCH_2), 39.2 (CH), 29.6 (2C, CH_2), 28.3 (CH_2), 25.6 (2C, CH_2), 24.7 (CH_3), 17.1 (CH_3), 14.5 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 435.07 ($[M+H]^+$, 100%); Anal. ($C_{26}H_{34}N_4O_2$): calcd, C 71.86, H 7.89, N 12.89; found, C 71.46, H 7.62, N 13.27.

5.2.7. 2-(2-(4-Cyclohexylmethoxyphenyl)-5,7-dimethylpyrazolo [1,5-a]pyrimidin-3-yl)-N,N-diethylacetamide (20)

Treating **13** with (bromomethyl)cyclohexane (105 μL, 0.75 mmol, 1.5 equiv.) according to the general procedure gave **20** as a colorless crystalline solid (193 mg, 86%). m.p. 141–143 °C; R_f 0.42 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74 (2H, d, J = 8.5 Hz, ArH), 6.96 (2H, d, J = 8.5 Hz, ArH), 6.49 (1H, s, ArH), 3.92 (2H, s, $ArCH_2$), 3.79 (2H, d, J = 6.5 Hz, OCH_2), 3.49 (2H, q, J = 7.0 Hz, NCH_2), 3.41 (2H, q, J = 7.2 Hz, NCH_2), 2.73 (3H, s, $ArCH_3$), 2.54 (3H, s, $ArCH_3$), 1.89–1.69 (6H, m), 1.34–1.22 (3H, m), 1.20 (3H, t, J = 7.0 Hz, CH_2CH_3), 1.11 (3H, t, J = 7.0 Hz, CH_2CH_3), 1.10–1.02 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2 (C=O), 159.7 ($C_{quat.}$), 157.5 ($C_{quat.}$), 155.3 ($C_{quat.}$), 147.6 ($C_{quat.}$), 145.0 ($C_{quat.}$), 130.0 (2C, CH), 126.1 ($C_{quat.}$), 114.7 (2C, CH), 108.2 (CH), 100.9 ($C_{quat.}$), 73.7 (OCH_2), 42.4 (NCH_2), 40.7 (NCH_2), 37.8 (CH), 30.1 (2C, CH_2), 28.3 (CH_2), 26.7 (CH_2), 26.0 (2C, CH_2), 24.6 (CH_3), 17.1 (CH_3), 14.5 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 449.13 ($[M+H]^+$, 100%); Anal. ($C_{27}H_{36}N_4O_2$): calcd, C 72.29, H 8.09, N 12.49; found, C 72.38, H 8.32, N 12.43.

5.2.8. 2-(2-(4-(Benzyl)oxyphenyl)-5,7-dimethylpyrazolo[1,5-a] pyrimidin-3-yl)-N,N-diethylacetamide (21)

Treating **13** with benzyl bromide (90 μL, 0.75 mmol, 1.5 equiv.) according to the general procedure gave **21** as a colorless crystalline solid (191 mg, 86%). m.p. 151–153 °C; R_f 0.50 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78 (2H, d, J = 8.5 Hz, ArH), 7.45 (2H, d, J = 7.5 Hz, ArH), 7.40–7.37 (2H, m, ArH), 7.34–7.31 (1H, m, ArH), 7.05 (2H, d, J = 8.5 Hz, ArH), 6.50 (1H, s, ArH), 5.12 (2H, s, OCH_2), 3.91 (2H, s, $ArCH_2$), 3.50 (2H, q, J = 7.2 Hz, NCH_2), 3.40 (2H, q, J = 7.2 Hz, NCH_2), 2.74 (3H, s, $ArCH_3$), 2.54 (3H, s, $ArCH_3$), 1.19 (3H, t, J = 7.2 Hz, CH_2CH_3), 1.11 (3H, t, J = 7.2 Hz, CH_2CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.3 (C=O), 159.2 ($C_{quat.}$), 157.6 ($C_{quat.}$), 155.2 ($C_{quat.}$), 147.9 ($C_{quat.}$), 144.8 ($C_{quat.}$), 137.1 ($C_{quat.}$), 130.2 (2C, CH), 128.7 (2C, CH), 128.1 (CH), 127.6 (2C, CH), 126.8 ($C_{quat.}$), 115.1 (2C, CH), 108.3 (CH), 101.0 ($C_{quat.}$), 70.2 (OCH_2), 42.5 (NCH_2), 40.7 (NCH_2), 28.3 (CH_2), 24.8 (CH_3), 17.1 (CH_3), 14.5 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 443.07 ($[M+H]^+$, 100%); Anal. ($C_{27}H_{30}N_4O_2$): calcd, C 73.28, H 6.83, N 12.66; found, C 73.28, H 6.82, N 12.61.

5.2.9. 2-(2-(4-((2-Fluorobenzyl)oxy)phenyl)-5,7-dimethylpyrazolo [1,5-a]pyrimidin-3-yl)-N,N-diethylacetamide (22)

Treating **13** with 2-fluorobenzyl bromide (90 μL, 0.75 mmol, 1.5 equiv.) according to the general procedure gave **22** as a colorless crystalline solid (208 mg, 90%). m.p. 146–148 °C; R_f 0.49 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (2H, d, J = 8.8 Hz, ArH), 7.51 (1H, td, J = 7.4, 1.2 Hz, ArH), 7.32–7.28 (1H, m, ArH), 7.16 (1H, td, J = 7.4, 1.2 Hz, ArH), 7.11–7.05 (1H, m, ArH), 7.06 (2H, d, J = 8.5 Hz, ArH), 6.49 (1H, s, ArH), 5.19 (2H, s, OCH_2), 3.91 (2H, s, $ArCH_2$), 3.50 (2H, q, J = 7.2 Hz, NCH_2), 3.40 (2H, q, J = 7.2 Hz, NCH_2), 2.73 (3H, s, $ArCH_3$), 2.53 (3H, s, $ArCH_3$), 1.19 (3H, t, J = 7.2 Hz, CH_2CH_3), 1.11 (3H, t, J = 7.2 Hz, CH_2CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.2 (C=O), 160.6 (d, $^1J_{CF}$ = 247.0 Hz, $C_{quat.}$), 158.8 ($C_{quat.}$), 157.6 ($C_{quat.}$), 155.1 ($C_{quat.}$), 147.8 ($C_{quat.}$), 144.8 ($C_{quat.}$), 130.2 (2C, CH), 129.8 (CH), 129.7 (d, $^3J_{CF}$ = 5.2 Hz, CH), 127.0 ($C_{quat.}$), 124.4 (d, $^4J_{CF}$ = 3.6 Hz, CH), 124.2 ($C_{quat.}$), 115.4 (d, $^2J_{CF}$ = 21.0 Hz, CH), 115.0 (2C, CH), 108.3 (CH), 101.0 ($C_{quat.}$), 63.8 (d, $^3J_{CF}$ = 4.5 Hz, OCH_2), 42.4 (NCH_2), 40.7 (NCH_2), 28.2 (CH_2), 24.7 (CH_3), 17.0 (CH_3), 14.5 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 461.07 ([M+H]$^+$, 100%); Anal. ($C_{27}H_{29}N_4O_2F$): calcd, C 70.41, H 6.35, N 12.17; found, C 70.42, H 6.45, N 12.11.

5.2.10. 2-(2-(4-((3-Fluorobenzyl)oxy)phenyl)-5,7-dimethylpyrazolo [1,5-a]pyrimidin-3-yl)-N,N-diethylacetamide (23)

Treating **13** with 3-fluorobenzyl bromide (90 μL, 0.75 mmol, 1.5 equiv.) according to the general procedure gave **23** as a colorless crystalline solid (189 mg, 82%). m.p. 151–153 °C; R_f 0.53 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, d, J = 8.8 Hz, ArH), 7.37–7.31 (1H, m, ArH), 7.21–7.16 (2H, m, ArH), 7.04 (2H, d, J = 8.8 Hz, ArH), 6.99 (1H, dd, J = 8.4, 2.0 Hz, ArH), 6.49 (1H, s, ArH), 5.11 (2H, s, OCH_2), 3.91 (2H, s, $ArCH_2$), 3.50 (2H, q, J = 7.1 Hz, NCH_2), 3.40 (2H, q, J = 7.1 Hz, NCH_2), 2.73 (3H, s, $ArCH_3$), 2.53 (3H, s, $ArCH_3$), 1.19 (3H, t, J = 7.2 Hz, CH_2CH_3), 1.11 (3H, t, J = 7.2 Hz, CH_2CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.2 (C=O), 163.1 (d, $^1J_{CF}$ = 246.2 Hz, $C_{quat.}$), 158.8 ($C_{quat.}$), 157.6 ($C_{quat.}$), 155.0 ($C_{quat.}$), 147.8 ($C_{quat.}$), 144.8 ($C_{quat.}$), 139.8 (d, $^3J_{CF}$ = 7.2 Hz, $C_{quat.}$), 130.3 (d, $^3J_{CF}$ = 8.1 Hz, CH), 130.2 (2C, CH), 127.0 ($C_{quat.}$), 122.8 (d, $^4J_{CF}$ = 2.9 Hz, CH), 115.0 (2C, CH), 114.9 (d, $^2J_{CF}$ = 21.1 Hz, CH), 114.3 (d, $^2J_{CF}$ = 22.1 Hz, CH), 108.3 (CH), 101.0 ($C_{quat.}$), 69.3 (d, $^4J_{CF}$ = 1.8 Hz, OCH_2), 42.4 (NCH_2), 40.7 (NCH_2), 28.3 (CH_2), 24.7 (CH_3), 17.0 (CH_3), 14.4 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 461.07 ([M+H]$^+$, 100%); Anal. ($C_{27}H_{29}N_4O_2F$): calcd, C 70.41, H 6.35, N 12.17; found, C 70.50, H 6.12, N 12.00.

5.2.11. 2-(2-(4-((4-Fluorobenzyl)oxy)phenyl)-5,7-dimethylpyrazolo [1,5-a]pyrimidin-3-yl)-N,N-diethylacetamide (24)

Treating **13** with 4-fluorobenzyl bromide (95 μL, 0.75 mmol, 1.5 equiv.) according to the general procedure gave **24** as a colorless crystalline solid (186 mg, 81%). m.p. 104–106 °C; R_f 0.44 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (2H, d, J = 8.4 Hz, ArH), 7.43–7.40 (2H, m, ArH), 7.09–7.02 (4H, m, ArH), 6.49 (1H, s, ArH), 5.07 (2H, s, OCH_2), 3.91 (2H, s, $ArCH_2$), 3.50 (2H, q, J = 7.2 Hz, NCH_2), 3.40 (2H, q, J = 7.2 Hz, NCH_2), 2.73 (3H, s, $ArCH_3$), 2.53 (3H, s, $ArCH_3$), 1.20 (3H, t, J = 7.2 Hz, CH_2CH_3), 1.10 (3H, t, J = 7.2 Hz, CH_2CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.2 (C=O), 162.6 (d, $^1J_{CF}$ = 246.3 Hz, $C_{quat.}$), 158.9 ($C_{quat.}$), 157.6 ($C_{quat.}$), 155.1 ($C_{quat.}$), 147.7 ($C_{quat.}$), 144.9 ($C_{quat.}$), 132.9 (d, $^4J_{CF}$ = 3.2 Hz, CH), 130.2 (2C, CH), 129.4 (2C, d, $^3J_{CF}$ = 8.3 Hz, CH), 126.9 ($C_{quat.}$), 115.6 (2C, d, $^2J_{CF}$ = 21.5 Hz, CH), 115.0 (2C, CH), 108.3 (CH), 101.0 ($C_{quat.}$), 69.5 (OCH_2), 42.4 (NCH_2), 40.7 (NCH_2), 28.3 (CH_2), 24.7 (CH_3), 17.1 (CH_3), 14.5 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 461.07 ([M+H]$^+$, 100%); Anal. ($C_{27}H_{29}N_4O_2F$): calcd, C 70.41, H 6.35, N 12.17; found, C 70.47, H 6.40, N 12.01.

5.2.12. 2-(2-(4-((2-(Trifluoromethyl)benzyl)oxy)phenyl)-5,7-dimethylpyrazolo[1,5-a]pyrimidin-3-yl)-N,N-diethylacetamide (25)

Treating **13** with 2-(trifluoromethyl)benzyl bromide (179 mg, 0.75 mmol, 1.5 equiv.) according to the general procedure gave **25** as a colorless crystalline solid (222 mg, 87%). m.p. 103–105 °C; R_f 0.48 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80–7.69 (4H, m, ArH), 7.56 (1H, t, J = 7.6 Hz, ArH), 7.41 (1H, t, J = 7.6 Hz, ArH), 7.04 (2H, d, J = 8.8 Hz, ArH), 6.50 (1H, s, ArH), 5.30 (2H, s, OCH_2), 3.91 (2H, s, $ArCH_2$), 3.50 (2H, q, J = 7.2 Hz, NCH_2), 3.40 (2H, q, J = 7.2 Hz, NCH_2), 2.73 (3H, s, $ArCH_3$), 2.53 (3H, s, $ArCH_3$), 1.19 (3H, t, J = 7.2 Hz, CH_2CH_3), 1.11 (3H, t, J = 7.2 Hz, CH_2CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.2 (C=O), 158.6 ($C_{quat.}$), 157.6 ($C_{quat.}$), 155.1 ($C_{quat.}$), 147.7 ($C_{quat.}$), 144.9 ($C_{quat.}$), 135.9 (CH), 132.3 ($C_{quat.}$), 130.2 (2C, CH), 128.7 (CH), 127.6 (CH), 127.4 (q, $^2J_{C\text{-}F}$ = 30.9 Hz, $C_{quat.}$), 127.1 ($C_{quat.}$), 125.8 (q, $^3J_{C\text{-}F}$ = 5.7 Hz, CH), 124.5 (q, $^1J_{C\text{-}F}$ = 252.6 Hz, CF_3), 115.1 (2C, CH), 108.3 (CH), 101.0 ($C_{quat.}$), 66.2 (q, $^4J_{C\text{-}F}$ = 3.1 Hz, OCH_2), 42.5 (NCH_2), 40.7 (NCH_2), 28.2 (CH_2), 24.7 (CH_3), 17.0 (CH_3), 14.5 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 511.07 ([M+H]$^+$, 100%); Anal. ($C_{28}H_{29}N_4O_2F_3$): calcd, C 65.87, H 5.73, N 10.97; found, C 65.64, H 5.66, N 10.56.

5.2.13. 2-(2-(4-((3-(Trifluoromethyl)benzyl)oxy)phenyl)-5,7-dimethylpyrazolo[1,5-a]pyrimidin-3-yl)-N,N-diethylacetamide (26)

Treating **13** with 3-(trifluoromethyl)benzyl bromide (115 μL, 0.75 mmol, 1.5 equiv.) according to the general procedure gave **26** as a colorless crystalline solid (177 g, 69%). m.p. 120–122 °C; R_f 0.45 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.80 (2H, d, J = 8.5 Hz, ArH), 7.73 (1H, s, ArH), 7.64 (1H, d, J = 7.5 Hz, ArH), 7.59 (1H, d, J = 7.5 Hz, ArH), 7.51 (1H, t, J = 7.5 Hz, ArH), 7.05 (2H, d, J = 8.5 Hz, ArH), 6.51 (1H, s, ArH), 5.16 (2H, s, OCH_2), 3.94 (2H, s, $ArCH_2$), 3.51 (2H, q, J = 7.2 Hz, NCH_2), 3.40 (2H, q, J = 7.2 Hz, NCH_2), 2.74 (3H, s, $ArCH_3$), 2.55 (3H, s, $ArCH_3$), 1.20 (3H, t, J = 7.2 Hz, CH_2CH_3), 1.11 (3H, t, J = 7.2 Hz, CH_2CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2 (C=O), 158.7 ($C_{quat.}$), 157.6 ($C_{quat.}$), 155.0 ($C_{quat.}$), 147.7 ($C_{quat.}$), 144.9 ($C_{quat.}$), 138.2 ($C_{quat.}$), 130.9 (q, $^2J_{C\text{-}F}$ = 31.9 Hz, $C_{quat.}$), 130.7 (CH), 130.2 (2C, CH), 129.2 (CH), 127.2 ($C_{quat.}$), 124.9 (q, $^3J_{C\text{-}F}$ = 3.8 Hz, CH), 124.20 (q, $^1J_{C\text{-}F}$ = 272.2 Hz, CF_3), 124.16 (q, $^3J_{C\text{-}F}$ = 3.8 Hz, CH), 115.0 (2C, CH), 108.3 (CH), 101.0 ($C_{quat.}$), 69.3 (OCH_2), 42.5 (NCH_2), 40.7 (NCH_2), 28.3 (CH_2), 24.7 (CH_3), 17.0 (CH_3), 14.5 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 511.07 ([M+H]$^+$, 100%); Anal. ($C_{28}H_{29}N_4O_2F_3$): calcd, C 65.87, H 5.73, N 10.97; found, C 65.69, H 5.71, N 10.83.

5.2.14. 2-(2-(4-((4-(Trifluoromethyl)benzyl)oxy)phenyl)-5,7-dimethylpyrazolo[1,5-a]pyrimidin-3-yl)-N,N-diethylacetamide (27)

Treating **13** with 4-(trifluoromethyl)benzyl bromide (179 mg, 0.75 mmol, 1.5 equiv.) according to the general procedure gave **27** as a colorless crystalline solid (199 mg, 78%). m.p. 145–147 °C; R_f 0.60 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.80 (2H, d, J = 8.5 Hz, ArH), 7.64 (2H, d, J = 8.0 Hz, ArH), 7.56 (2H, d, J = 8.0 Hz, ArH), 7.04 (2H, d, J = 8.5 Hz, ArH), 6.50 (1H, s, ArH), 5.17 (2H, s, OCH_2), 3.91 (2H, s, $ArCH_2$), 3.50 (2H, q, J = 7.0 Hz, NCH_2), 3.41 (2H, q, J = 7.0 Hz, NCH_2), 2.73 (3H, s, $ArCH_3$), 2.54 (3H, s, $ArCH_3$), 1.19 (3H, t, J = 7.2 Hz, CH_2CH_3), 1.10 (3H, t, J = 7.2 Hz, CH_2CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2 (C=O), 158.7 ($C_{quat.}$), 157.6 ($C_{quat.}$), 155.0 ($C_{quat.}$), 147.7 ($C_{quat.}$), 144.9 ($C_{quat.}$), 141.2 ($C_{quat.}$), 130.2 (2C, CH), 130.2 (q, $^2J_{C\text{-}F}$ = 32.4 Hz, $C_{quat.}$) 127.5 (2C, CH), 127.2 ($C_{quat.}$), 125.7 (q, $^3J_{C\text{-}F}$ = 3.8 Hz, CH), 124.2 (q, $^1J_{C\text{-}F}$ = 272.0 Hz, CF_3), 115.0 (2C, CH), 108.3 (CH), 101.0 ($C_{quat.}$), 69.2 (OCH_2), 42.5 (NCH_2), 40.7 (NCH_2), 28.3 (CH_2), 24.7 (CH_3), 17.0 (CH_3), 14.5 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 511.07 ([M+H]$^+$, 100%); Anal. ($C_{28}H_{29}N_4O_2F_3$): calcd, C 65.87, H 5.73, N 10.97; found, C 65.91, H 5.76, N 10.97.

5.2.15. 2-(2-(4-Phenethoxyphenyl)-5,7-dimethylpyrazolo[1,5-a] pyrimidin-3-yl)-N,N-diethylacetamide (28)

Treating **13** with 1-bromo-2-phenylethane (100 μL, 0.75 mmol, 1.5 equiv.) according to the general procedure gave **28** as a colorless crystalline solid (193 mg, 85%). m.p. 133–135 °C; R_f 0.42 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (2H, d, J = 8.5 Hz, ArH), 7.34–7.29 (4H, m, ArH), 7.26–7.23 (1H, m, ArH), 6.98 (2H, d, J = 8.5 Hz, ArH), 6.49 (1H, s, ArH), 4.22 (2H, t, J = 7.3 Hz, OCH_2), 3.91 (2H, s, $ArCH_2$), 3.50 (2H, q, J = 7.2 Hz, NCH_2), 3.41 (2H, q, J = 7.2 Hz, NCH_2), 3.12 (2H, t, J = 7.3 Hz, $OCH_2C\underline{H}_2$), 2.74 (3H, s, $ArCH_3$), 2.54 (3H, s, $ArCH_3$), 1.20 (3H, t, J = 7.2 Hz, $CH_2C\underline{H}_3$), 1.11 (3H, t, J = 7.2 Hz, $CH_2C\underline{H}_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2 (C═O), 159.1 ($C_{quat.}$), 157.6 ($C_{quat.}$), 155.2 ($C_{quat.}$), 147.7 ($C_{quat.}$), 144.9 ($C_{quat.}$), 138.3 ($C_{quat.}$), 130.1 (2C, CH), 129.1 (2C, CH), 128.6 (2C, CH), 127.6 (CH), 126.5 (CH), 125.7 ($C_{quat.}$), 114.8 (2C, CH), 108.2 (CH), 100.9 ($C_{quat.}$), 68.8 (OCH_2), 42.4 (NCH_2), 40.7 (NCH_2), 35.9 (CH_2), 28.3 (CH_2), 24.7 (CH_3), 17.0 (CH_3), 14.5 (CH_3), 13.2 (CH_3); LRMS (+ESI) m/z 457.13 ($[M+H]^+$, 100%); Anal. ($C_{28}H_{32}N_4O_2$): calcd, C 73.66, H 7.06, N 12.27; found, C 73.63, H 7.25, N 12.24.

5.3. Binding studies

Mitochondria were prepared as previously described [33,38], with minor modifications as described below, from kidneys of male Wistar rats killed by cervical dislocation. Kidneys were homogenized in 20 volumes of ice-cold 50 mM Tris/HCl, pH 7.4, 0.32 M sucrose and 1 mM EDTA (buffer A), containing protease inhibitors (160 μg/mL benzamidine, 200 μg/mL bacitracine and 20 μg/mL soybean trypsin inhibitor) with a Teflon pestle in a glass homogenizer and centrifuged at 600 *g* for 10 min at 4 °C. The resulting supernatant was centrifuged at 10,000 *g* for 10 min at 4 °C. The pellet was then resuspended in 20 volumes of ice-cold buffer A and centrifuged again at 10,000 *g* for 10 min at 4 °C. The crude mitochondrial pellet was frozen at −20 °C until the time of assay or incubated with 0.6 nM [^{3}H]PK11195 in 50 mM Tris/HCl, pH 7.4 (buffer B), with a range of concentrations of the tested compounds (0.1 nM–10 μM) in a total volume of 0.5 mL for 90 min at 4 °C. The incubation was terminated by dilution to 5 mL with ice-cold buffer B, followed immediately by rapid filtration through glass-fiber filters (Whatman GF/C). The filters were then washed with buffer B (2.5 mL) and the amount of radioactivity retained on the filters was determined using a Packard 1600 TR liquid scintillation counter at 66% efficiency. Non-specific binding was estimated in each case in the presence of 1 μM of unlabelled PK11195. The IC_{50} values were determined and K_i values were derived according to the equation previously derived [39]. Protein concentration was estimated by the method of Lowry and colleagues [40] with bovine serum albumin (BSA) as standard.

5.4. Cell culture

Rat C6 glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2/95% air at 37 °C.

5.5. Steroidogenesis assay

C6 cells were seeded in 96-well plates at a density of 1×10^4 cells/well in a final volume of 0.1 mL. Prior to measurement of pregnenolone production, the cells were washed three times with a simple salts aqueous medium consisting of 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM glucose, 10 mM HEPES/NaOH, pH 7.4, plus 0.1% BSA. During experiments, cells were incubated with this simple salts medium in an air incubator at 37 °C. In order to measure pregnenolone secreted into the medium, its further metabolism was blocked by the addition of trilostane (25 μM) and SU 10603 (10 μM) (inhibitors of 3β-hydroxysteroid dehydrogenase and 17α-hydroxylase, respectively) to the simple salts aqueous medium, as previously described [35]. The addition of the compounds to the C6 cells was made by the complete change of the simple salts medium to a medium containing the appropriate concentration (40 μM) of each compound. The final concentration of DMSO was constant for all the wells within each experiment and did not exceed 0.5% (v/v), a concentration which, on its own, had no effect on steroid production. At the end of the incubation period (2 h), the cell medium was used in an enzyme immunoassay for the direct quantitative determination of pregnenolone, under the conditions recommended by the supplier (Pregnenolone ELISA, the EiAsy Way, IBL Hamburg, Germany). Cell protein concentration was measured according to a previously described method [38].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.02.004.

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