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Interaction of novel condensed triazine derivatives with central and peripheral type benzodiazepine receptors: synthesis, in vitro pharmacology and modelling

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Abstract

Structurally related sets of triazino-quinoline, triazino-isoquinoline and pyrido-triazine derivatives were synthesised and their binding interactions with central (CBR)- and peripheral-type (PBR) benzodiazepine binding sites have been characterised. Of 33 compounds tested, a new compound, 2-(4-methylphenyl)-3*H*- [1,2,4] triazino [2, 3-*a*] quinolin-3-one (**1 g**) showed the lowest CBR binding inhibition constant ($K_i = 42 \pm 9$ nM) and the highest CBR over PBR selectivity (> 1300). All but the 4-methylphenyl (**1 g**) structural modifications decreased the affinity and selectivity of the parent compound, 2-phenyl-3*H*- [1,2,4]triazino[2,3-*a*]quinolin-3-one (**1d**) ($K_i = 69 \pm 9$ nM, selectivity > 890). Molecular interactions between selected ligands (standards and triazine derivatives) and $\alpha_1\gamma_2$ subunit-interface residues in a $GABA_A$ receptor extracellular domain homology model have been calculated. Comparing data with calculations confirmed hydrogen bonding to γ_2Thr142 and hydrophobic interaction with α_1His101 as being essential for high-affinity CBR binding.

Keywords: Ring closure; Cyclocondensation; Condensed triazine derivatives; [^{3}H]-flunitrazepam binding; [^{3}H]-PK 11195 binding

1. Introduction

The benzodiazepine receptor has been classified as central- (CBR) and peripheral-type (PBR) [1]. The CBR, located allosterically at the interface formed by the α and γ subunits of the chloride ion channel forming $GABA_A$ receptor oligomer [2,3] has long been implicated as a target for the treatment of anxiety, sleeplessness and epilepsy [4,5]. The PBR, initially discovered in peripheral tissues [6], distinguished from the CBR by its structure, function, sub-cellular localisation, tissue distribution and pharmacological profile [1]. The PBR is involved in several physiological phenomena like steroid and heme biosynthesis, immunomodulation and cell proliferation.

CBR and PBR interact with several structural classes of ligands (Fig. 1). CBR binds imidazo-1,4-benzodiazepines, 4-hydroxiquinolines, imidazoquinolines, pyrrolopyrazines, triazolopyridazines [7], pyrazoloquinolines [7,8], β-carbolines [7,9] and naturally occurring flavones and their synthetic derivatives [7,10–12]. PBR interacts with 1,4-benzodiazepines, isoquinoline carboxamides, indoleacetamides, porphyrins, dihydropyridines [1], indolyl glyoxylamides [13], imidazopyridines, and pyrrolobenzoxazepines [14].

Abbreviations: CBR, central-type benzodiazepine binding site; Flunitrazepam, 5-(2-fluorophenyl)-1-methyl-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one; $K_{i,FLU}$, inhibition constant for [^{3}H]-flunitrazepam binding; $K_{i,PK}$, inhibition constant for [^{3}H]-PK 11195 binding; LRAA, ligand recognising amino acids; PBR, peripheral-type benzodiazepine binding site; PK 11195, N-(sec-butyl)-1-(2-chlorophenyl)-N-methylisoquinoline-3-carboxamide; Ro 5-4864, 7-chloro-5-(4-chlorophenyl)-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one; Ro15-1788 (flumazenil), ethyl 8-fluoro-5-methyl-6-oxo-3a,4,5,6-tetrahydro-3H-imidazo[1,5-a] [1,4]benzodiazepine-3-carboxylate; TRIS, tris(hydroxymethyl)aminomethane.

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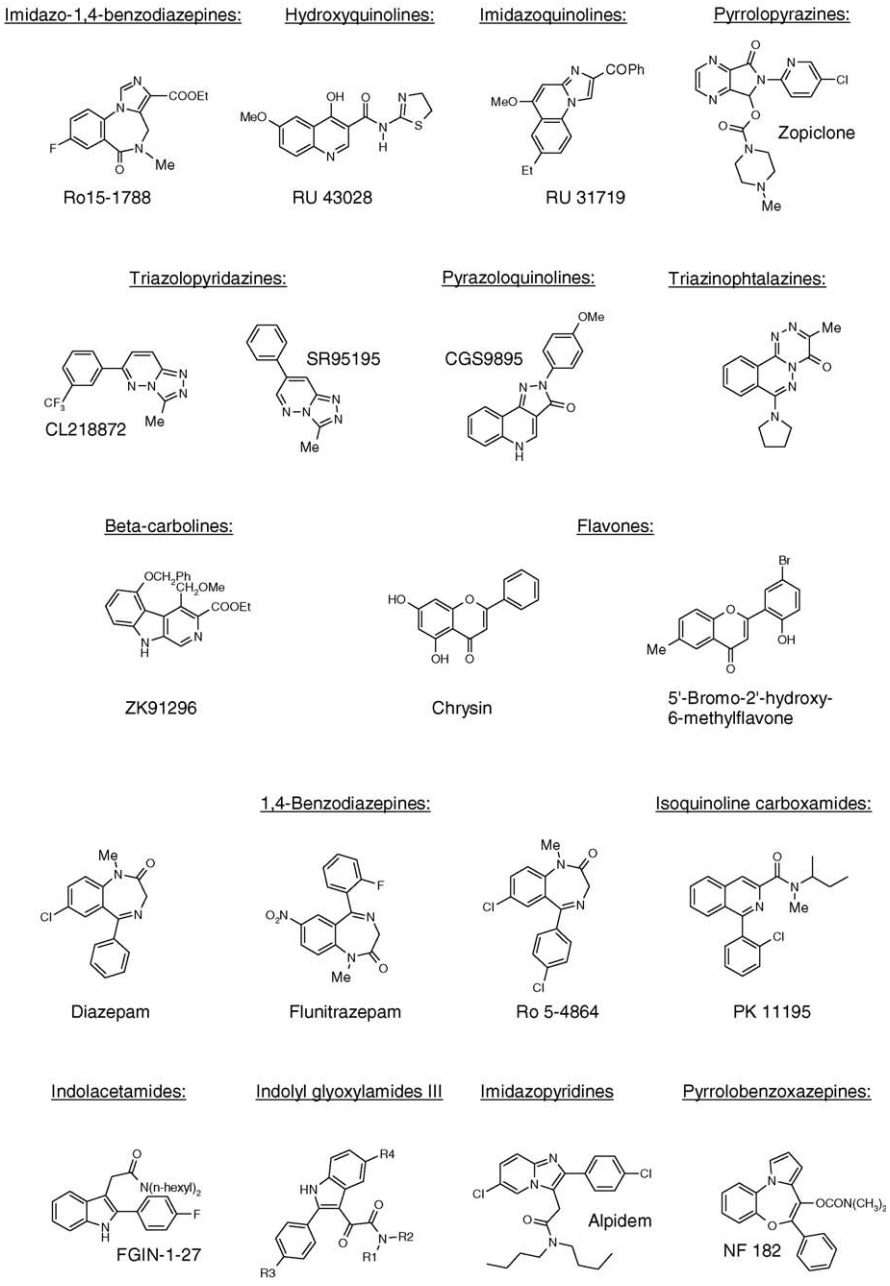


Fig. 1. Structural classes of ligands that interact with CBR and PBR.

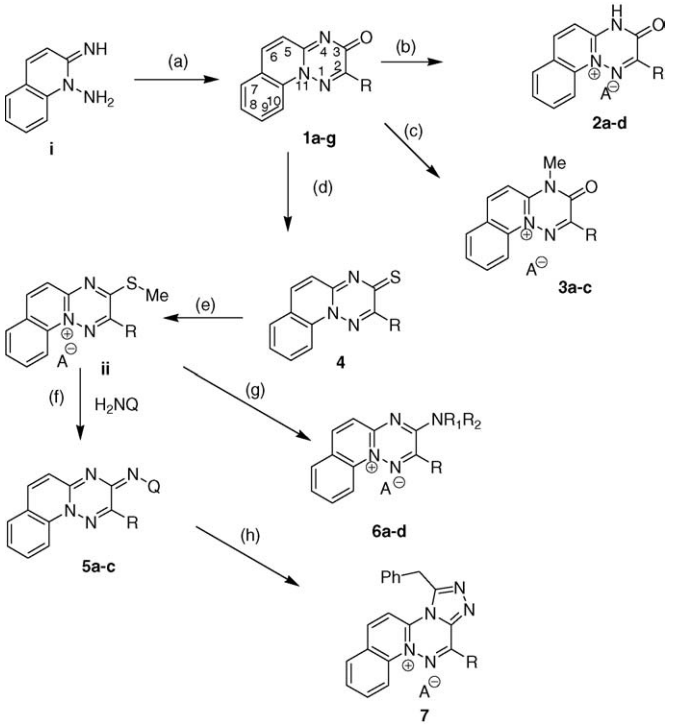
7-chloro-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (diazepam, Fig. 1.) [1] and 5-(2-fluorophenyl)-1-methyl-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one (flunitrazepam, Fig. 1.) [15] show affinity for both, CBR and PBR. Ligands used to distinguish between these two receptors are: ethyl 8-fluoro-5-methyl-6-oxo-3a,4,5,6-tetrahydro-3H-imidazo [1,5-*a*] [1,4]benzodiazepine-3-carboxylate (Ro15-1788, flumazenil, Fig. 1.) for CBR [1] and 7-chloro-5-(4-chlorophenyl)-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (Ro 5-4864, Fig. 1.) and N-(sec-butyl)-1-(2-chlorophenyl)-N-methylisoquinoline-3-carboxamide (PK 11195, Fig. 1.) for PBR [1]. CBR vs. PBR binding site-selectivity has been characterised in the context of PBR-selective compounds [13,14,16]. Surprisingly, PBR binding data are usually not available for CBR ligands, including several classes of quinolines [7,8] and different α subunit subtype-selective compounds [17,18], planned for the development of more specific anxiolytic drugs [5,19].

We report here the synthesis and pharmacological characterisation of structurally related sets of 33 condensed triazine (triazino-quinoline, triazino-isoquinoline and pyrido-triazine) derivatives, including 19 new compounds, by measuring displacement of [^{3}H]-flunitrazepam and [^{3}H]-PK 11195 from their specific binding sites in brain membrane homogenates. Predictive structural chemistry was attempted by application of a homology model of the $GABA_A$ receptor extracellular domain [20]. CBR-ligand interactions disclosed by docking different types of reference compounds, including 5′-bromo-2′-hydroxy-6-methylflavone, into the homology model were compared

to those predicted by point mutation and photoaffinity labelling data as well as by the refined pharmacophore model for flavone derivatives binding [11], and were used to find more effective novel triazine derivatives.

2. Chemistry

The tested compounds were synthesised starting from 2-iminoquinolin-1(2H)-amine (**i**), 1-iminoisoquinolin-2(1H)-amine (**iii**) or 1,2-diamino-pyridinium salt (**v**) as shown in Scheme 1., 2. and 3. The first step of the pathway to **1-7** (Scheme 1.) was the reaction of 2-iminoquinolin-1(2H)-amine (**i**) [21] with differently substituted ethyl oxo-acetates to give 3*H*- [1,2,4] triazino[2,3-*a*]quinolin-3-ones (**1**). Further transformations of **1** by treatment with acids or dimethyl sulphate led to the salts **2** and **3**, respectively. Thus the protonation and the methylation took place at position *N*-4. In order to obtain further functionalised derivatives, the oxo-function was exchanged for a thione by using P_2S_5 in pyridine to give **4**, which was reacted with dimethyl sulphate to afford methylthio compound (**ii**). The methylthio group of this product proved to be easily exchangeable by nucleophiles. Thus, primary amines and hydrazides gave imines (**5**) whereas secondary amines afforded quinolinium salts (**6**). Compound **5b** was converted to triazolo [4′,3′:4,5]triazino[2,3-*a*]quinolin-11-ium perchlorate (**7**) a well established ring closure methodology by using $POCl_3$. Synthesis of related isoquinoline derivatives (Scheme 2.) was carried out in similar manner as described above: reaction of 1-iminoisoquinolin-2(1H)-amine (**iii**) with ethyl oxo-acetates gave 2*H*-[1,2,4]triazino[3,2-*a*]isoquinolin-2-one [21] (**8**) and methylation of this compounds led to the methoxy derivative (**iv**) which was converted to amino substituted salts (**9**). Similar to the ring closure of **5b→7**, compound **9d** was also converted to the tetracyclic 1-benzyl-4-phenyl [1,2,4]triazolo[4′,3′:4,5] [1,2,4]triazino[3,2-*a*]isoquinolin-6-ium perchlorate (**10**). Pyridine derivatives tested has been described elsewhere [22,23]. In brief, 1,2-diamino-pyridinium salt (**v**) was used as the starting compound and selective ring closures were carried out to give **11**, **12**, **13** and **14** derivatives (Scheme 3.).



Scheme 1. Synthetic routes to triazino-quinoline derivatives **1-7**: a) ethyl oxo-acetates, acetonitrile, rt, 4 h (**1a**: R = H; **1b**: R = Me; **1c**: R = COOEt; **1d**: R = Ph; **1e**: R = 4-Cl-C_6H_4; **1f**: R = 4-F-C_6H_4; **1 g**: R = 4-Me-C_6H_4); (b) acetonitrile, acids, diethyl ether, rt (**2a**: R = Ph, A = $EtSO_3$; **2b**: R = Ph, A = BF_4; **2c**: R = Ph, A=ClO_4; **2d**: R = 4-Me-C_6H_4, A = BF_4); (c) acetonitrile, dimethyl sulphate, 80 C° (**3a**: R = Ph, A = $EtSO_3$; **3b**: R = Ph, A=BF_4; **3c**: R = 4-Me-C_6H_4, A = BF_4); (d) pyridine, P_2S_5 (**4**: R = Ph); (e) acetonitrile, dimethyl sulphate, rt (**ii**: R= Ph); (f) acetonitrile, amine, rt (**5a**: R= Ph, Q = CH_2-Ph; **5b**: R = Ph, Q = $NHCOCH_2$-Ph; **5c**: R = Ph, Q = H); (g) acetonitrile, amine, rt (**6a**: R = Ph, NR_1R_2 = $NH(CH_2)_2N(CH_3)_2$, A = Br; **6b**: R = Ph, NR_1R_2 = morpholine, A = ClO_4; **6c**: R = Ph, NR_1R_2 = $NH(CH_2)_2OH$, A = $EtSO_3$; **6d**: R = Ph, NR_1R_2 = NH_2, A = ClO_4); (h) $POCl_3$, reflux, 1 h (**7**: R = Ph, A = ClO_4).

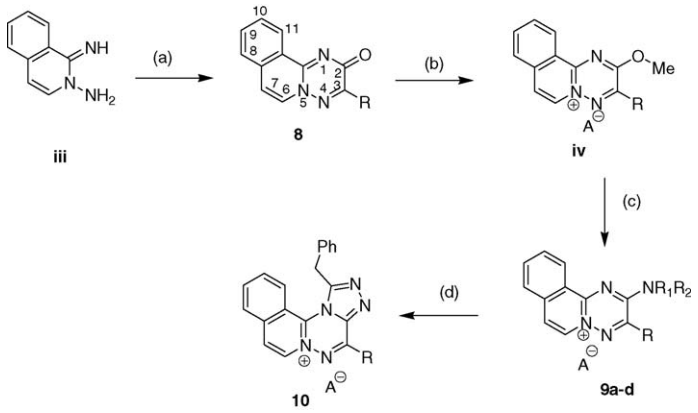
3. Pharmacology

3.1. In vitro affinity screening

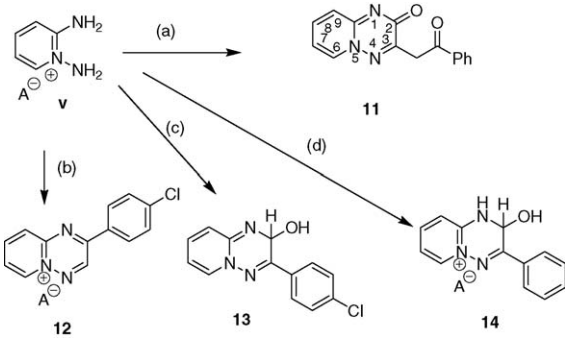
In vitro affinity screening of structurally related sets of 33 condensed triazine (triazino-quinoline, triazino-isoquinoline and pyrido-triazine) derivatives, including 19 new compounds, have been performed by measuring displacement of [^{3}H]-flunitrazepam and [^{3}H]-PK 11195 from their specific binding sites in brain membrane homogenates.

[^{3}H]-flunitrazepam and [^{3}H]-PK 11195 binding tests were characterised by inhibition constant for Ro 15-1788, $K_{i,FLU}$ = 0.9 ± 0.1 nM and PK 11195, $K_{i,PK}$=0.6 ± 0.1 nM, respectively (Table 1.). The $K_{i,FLU}$ and $K_{i,PK}$ values were lower than the values published earlier ($K_{i,FLU}$=3.5 ± 0.2 nM [24], $K_{i,FLU}$ = 5.0 ± 0.3 nM [12], $K_{i,PK}$ = 2.9 ± 0.1 nM [25] and $K_{i,PK}$ = 2.0 ± 0.3 nM [26]), indicating better test conditions. The variations can be accounted for differences of biological preparations and specific activity of radioligands used [12,24–26]. In addition, high-affinity binding of Ro 15-1788 and PK 11195 suggest, that sites specifically labelled by [^{3}H]-flunitrazepam and [^{3}H]-PK 11195 in our tests were of the CBR and PBR type, respectively. Ro 15-1788 showed a >72,000-fold and PK 11195 a <0.00004-fold $K_{i,PK}/K_{i,FLU}$ selectivity in our tests. By comparison, the CBR-specific agonist, clonazepam was seen to be 25,000 times more effective ($K_{i,Ro\ 5\text{-}4864}/K_{i,diazepam}$) in inhibiting [^{3}H]-diazepam than [^{3}H]-Ro 5-4864 binding to rat brain membranes [27]. Hirsch and his co-workers found PK 11195 to show a 0.0016-fold $K_{i,Ro\ 5\text{-}4864}/K_{i,FLU}$ selectivity [15] using membrane preparations from whole rat brain and rat kidney mitochondria for evaluation of CBR and PBR affinities, respectively.

Further information on the experimental execution is outlined in the experimental Section 6.2.



Scheme 2. Synthetic routes to triazino-isoquinoline derivatives **8-10**: (a) ethyl oxo-acetate, acetonitrile, rt, 4 h (**8**: R = H); (b) acetonitrile, dimethyl sulphate, reflux (**iv**: = Ph); (c) acetonitrile, amine, rt (**9a**: R = Ph, $NR_1R_2 = NH_2$ A = $EtSO_3$; **9b**: R = Ph, $NR_1R_2 = NH(CH_2)_3N(CH_3)_2$, A = $EtSO_3$; **9c**: R = H, $NR_1R_2 = NH_2$, A = Br; **9d**: R = CONH-benzyl, NR_1R_2= NH-benzyl, A = Br); (d) $POCl_3$, reflux, 1 h (**10**: R = Ph, A = ClO_4).



Scheme 3. Synthetic routes to pyrido-triazines **11-14**: (a) see reference[22] (**11**); (b) 4-chloro-phenylglyoxal, methanol, $HClO_4$, rt (**12**: A = $EtSO_3$); (c) 4-chloro-phenylglyoxal, methanol, rt (**13**); (d) phenylglyoxal, acetonitrile, $HClO_4$, rt (**14**: A = ClO_4).

3.2. In silico affinity screening: validation of a $GABA_A$ receptor homology model

Based on the crystal structure of the acetylcholine binding protein, a pentameric $GABA_A$ receptor with $\alpha_1{}^2\beta_2{}^2\gamma_2$ subunit composition was modelled recently by Ernst et al. [20]. The α_1 and γ_2 peptide chains embracing the CBR were cut out from this model and this dimeric construct (ErnstDC model) was used for affinity screening.

To test the applicability of the ErnstDC model for the present problem, extensive docking calculations were performed by using the chiral conformers [28] of the non-selective agonist flunitrazepam and the CBR-selective antagonist Ro 15-1788. Ligand recognising amino acids (LRAA, defined as those residues which have at least one atom closer than 3 Å to any ligand atom), hydrogen bonding and hydrophobic interactions were determined. The LRAA sets obtained by docking the M conformers [28] of flunitrazepam and Ro 15-1788 into the ErnstDC model were found to be consistent with available point-mutation [29] and photoaffinity labelling [30–33] results. Specifically, the *N*-methyl group of the M conformer of flunitrazepam was found to be proximal to α_1His101 [31,32]. Similarly, photolabelling with an azide group of Ro15-4513 - in a position equivalent to that of the fluorine atom in Ro 15-1788 - showed that it is proximal to α_1Tyr209 [33], and docking results confirmed this for the M conformer of Ro 15-1788. These findings indicated that the ErnstDC model could account for the preference of the M over the P conformer [34]. Furthermore, mutagenesis experiments [35,36] showed α_1His101 as part of the CBR binding site. Additional point mutation studies identified α_1 residues implicated in LRAA set include Tyr159, Thr162, Gly200, Thr206, Tyr209, and Val211 [37–40]. In the γ_2 subunit, Phe77 [41,42], Met130 [41,42], the domains Lys41-Trp82 and Arg114-Asp161 [43], and most recently Met57, Tyr58, and Ala79 [29] were identified. All the above residues appear in the LRAA set obtained by docking into the ErnstDC model.

As an additional test, comparisons were made with the hydrogen bonding and hydrophobic interactions predicted by the ErnstDC model with the refined pharmacophore model for flavone derivatives binding [11]. The high-affinity flavone derivative, 5′-bromo-2′-hydroxy-6-methylflavone (Fig. 1.), designed [11] on the basis of the refined pharmacophore, was also docked into the CBR. The hydrogen bonding and hydrophobic interactions of the resulting complex revealed a close matching of the refined pharmacophore and the ErnstDC model.

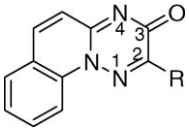
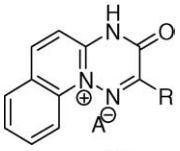
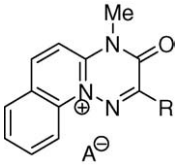
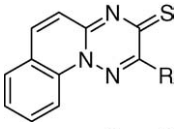
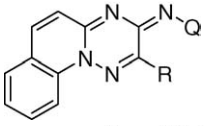
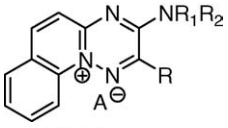
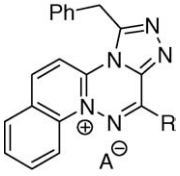
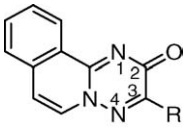
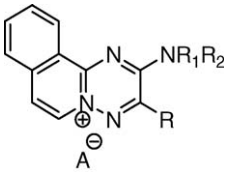
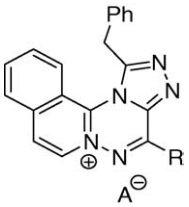
Further information on the modelling procedure is outlined in the experimental Section 6.3.

4. Results and discussion

4.1. In vitro affinity screening

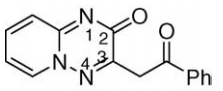
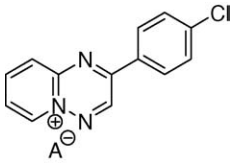
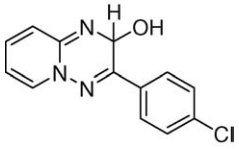
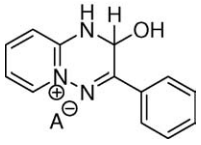
Considering the triazino-quinoline skeleton, the simultaneous presence of substituents at position 2 and 3 showed to be important for both, affinity and selectivity (Table 1.). The parent compound **1d**, with a phenyl ring in position 2 and oxo group in position 3 inhibited [^{3}H]-flunitrazepam binding with a K_i value of 0.069±0.009 μM, and showed >890-fold $K_{i,PK}/K_{i,FLU}$ selectivity (Table 1.). The inhibition constant was substantially increased upon halogen substitution ($K_{i,FLU}$ = 0.19 ± 0.03 μM for **1e**, and 0.42±0.24 μM for **1f**, see Ta-

Table 1
Effective concentration of triazino-quinolines, triazino-isoquinolines and pyrido-triazines to displace [^{3}H]-flunitrazepam and [^{3}H]-PK 11195 from specific binding sites (N=2-4)

Compound		Effective concentration		$K_{i,PK}/K_{i,FLU}$
		$K_{i,FLU}$[a] (μM)	$K_{i,PK}$[a] (μM)	
Triazino-quinolines				
	**1a** (R = H)	> 30	> 70	–
	1b (R = Me)	5.7 ± 0.8	> 70	> 10
	1c (R = COOEt)	2.1 ± 0.1	> 70	> 30
	1d (R = Ph)	0.069 ± 0.009	> 70	> 890
	1e (R = 4-Cl-C_6H_4)	0.19 ± 0.03	> 70	> 330
	1f (R = 4-F-C_6H_4)	0.42 ± 0.24	> 70	> 106
	1 g (R = 4-Me-C_6H_4)	0.042 ± 0.009	> 70	> 1300
	**2a** (R = Ph, A = $EtSO_3$)	0.11 ± 0.02	> 70	> 540
	2b (R = Ph, A = BF_4)	0.069 ± 0.018	> 70	> 800
	2c (R = Ph, A = ClO_4)	0.096 ± 0.003	> 70	> 700
	2d (R = 4-Me-C_6H_4, A=BF_4)	0.084 ± 0.009	21 ± 7[b]	250
	**3a** (R = Ph, A = $EtSO_3$)	> 30	> 70	–
	3b (R = Ph, A = BF_4)	1.9 ± 0.3	> 70	> 31
	3c (R = 4-Me-C_6H_4, A = BF_4)	1.0 ± 0.2	> 70	> 58
	**4** (R = Ph)	7.5 ± 0.9	> 70	> 8
	**5a** (R = Ph, Q = CH_2Ph)	2.3 ± 0.2	> 70	> 28
	5b (R = Ph, Q=$NHCOCH_2Ph$)R27	14 ± 1	28 ± 14[b]	2
	5c (R = Ph, Q = H) R28	0.23 ± 0.02	> 70	> 280
	**6a** (NR_1R_2 = $NH(CH_2)_2NMe_2$)	> 30	> 70	–
	6b (NR_1R_2 = morpholine)R23	> 30	> 70	–
	6c (NR_1R_2 = $NH(CH_2)_2OH$)	> 30	> 70	–
	6d (NR_1R_2 = NH_2)	1.9 ± 0.2	> 70	> 31
	**7** (R = Ph, A = ClO_4)	0.58 ± 0.05	16 ± 1	28
Triazino-isoquinolines				
	**8** (R = H)	> 30	> 70	–
	**9a** (R = Ph, NR_1R_2 = NH_2)	> 30	> 70	–
	9b (NR_1R_2=$NH(CH_2)_3NMe_2$)	> 30	> 70	–
	9c (R = H, NR_1R_2 = NH_2)	> 30	> 70	–
	9d (R = CONH-benzyl, NR_1R_2 = NH-benzyl)	0.47 ± 0.11	> 70	> 120
	**10** (R = Ph, A = ClO_4)	> 30	8.8 ± 1.2	< 0.30

(continued)

Table 1 *(continued)*

Compound		Effective concentration		$K_{i,PK}/K_{i,FLU}$
		$K_{i,FLU}$[a] (μM)	$K_{i,PK}$[a] (μM)	
Pyrido-triazines				
	**11**	> 30	> 70	-
	**12** (A = $EtSO_3$)	> 30	> 70	–
	**13**	> 30	> 70	–
	**14** (A = ClO_4)	> 30	> 70	–
	Ro 15-1788	0.00090 ± 0.00006	> 70	> 72000
	PK 11195	> 15	0.00060 ± 0.00007	< 0.00004

[a] $K_{i,FLU}$ for [^{3}H]-flunitrazepam and $K_{i,PK}$ for [^{3}H]-PK 11195 binding;
[b] Because of the limited solubility of the compound, the K_i value was estimated on the basis of parallel shifts of displacement curves obtained for **2d** and **5b**.

ble 1.). In contrast, 4′-methyl substitution (**1 g**) increased [^{3}H]-Flunitrazepam binding inhibition and $K_{i,PK}/K_{i,FLU}$ selectivity by a factor of about two ($K_{i,FLU}$ = 0.042±0.009 μM, $K_{i,PK}/K_{i,FLU}$ >1300; Table 1.).

Triazine ring modification, such as substitution of imino group (**5c**) for carbonyl in **1d** resulted in a decrease of binding inhibition ($K_{i,FLU}$ = 0.23±0.02 μM vs. $K_{i,FLU}$ = 0.069 ±0.009 μM, respectively) and $K_{i,PK}/K_{i,FLU}$ selectivity (>280, Table 1.). Inhibitory effects of imino derivatives, **5a** and **5b** decreased with increasing substituent size, which took effect some inhibition of [^{3}H]-PK 11195 binding in case of **5b**. Also, a five-membered triazole ring derivative (**7**) of **5c** showed inhibition of [^{3}H]-PK 11195 binding. Other imino derivatives (**6a, 6b** and **6c**) showed no significant binding inhibition (Table 1.). Substitution of thione (**4**) for carbonyl in **1d** led to a further decrease of the inhibitory effect ($K_{i,FLU}$ = 7.5 ±0.9 μM) and $K_{i,PK}/K_{i,FLU}$ selectivity (>8), when compounds **1d** or **5c** were compared (Table 1).

An about two orders of magnitude reduction of [^{3}H]-Flunitrazepam binding affinity was observed with the tetrafluoroborate salt form of the *N*-methyl derivative of **1 g** (**3b**: 1.9 ±0.3 μM, Table 1). By itself, the tetrafluroborate salt formation in **1d** and **1 g** (**2b** and **2d**, respectively) caused no change or a moderate decrease in the [^{3}H]-flunitrazepam binding affinity (Table 1.). This is contrasted to the perchlorate salt of **5c**, whereby salt formation (**6d**) led to one order of magnitude lower inhibitory effect ($K_{i,FLU}$=1.9±0.2 μM, Table 1.). Among compounds having an isoquinoline ring system (**8**, **9a-d**, **10**) only **9d**, bearing large substituents in position 2 and 3 (benzylamino and benzylaminocarbonyl, respectively) inhibited [^{3}H]-flunitrazepam binding ($K_{i,FLU}$=0.47±0.11 μM) with no effect on [^{3}H]-PK 11195 binding ($K_{i,PK}$>70 μM, Table 1). By contrast, compound **10**, also an isoquinoline with a bulky substituent in position 1, preferred the binding to [^{3}H]-PK 11195-labelled sites to some extent ($K_{i,PK}$=8.8±1.2 μM vs. $K_{i,FLU}$ >30 μM).

Neither pyrido-triazines derivatives inhibited [^{3}H]-flunitrazepam and [^{3}H]-PK 11195 binding.

4.2. In silico affinity screening of triazine derivatives using the ErnstDC homology model

Triazino-quinoline, **1d** was seen to be hydrogen bonded to γ_2Thr142 (Fig. 2.), and entered into hydrophobic interactions with the same residues as flunitrazepam and Ro 15-1788, i.e. γ_2Thr142, α_1Tyr159, α_1Gln203 and α_1Thr206. Three substitutions were made at the 4′ phenyl position in compound **1d**: the hydrogen atom, which was seen to be proximal to α_1His101 by docking, was replaced by chlorine (**1e**), fluorine (**1f**) and a methyl group (**1 g**). The fluorine atom in **1f** was seen to be strongly hydrogen bonded to α_1His101. In case of 4′-Cl substitution (**1e**), the phenyl group was found to be rotated by about 90 degrees, as compared to **1d** and **1f**, thus avoiding contact with α_1His101. By contrast, docking revealed that the 4′-methyl group entered into hydrophobic interaction with α_1His101. The above findings are in accordance with that obtained with flunitrazepam and Ro 15-1788: hydrophobic interaction with α_1His101 is essential, and preferred over the polar one. Compound **5c** was found to dock similarly to **1d**, with not much difference in hydrogen bonding or surrounding residue pattern. The thione derivative, **4** also docked similarly to **1d**, with the difference that both the pendant phenyl group of the

ligand and the aromatic ring of α_1Tyr159 were rotated to a nearly parallel alignment leading to increased π-π interaction, which might explain the difference in binding affinity. The AutoDock procedure gave no useful hint of the binding site for **5b,** the relatively populated (≈10%) clusters gave positions way off the binding site, and all the other clusters contained single conformations wildly differing from each other. Molecular modelling studies showed the absence of hydrogen bonding between **3b** and the γ_2Thr142, in contrast with the presence of hydrogen bonding to the γ_2Thr142 residue observed with the reference compounds, flunitrazepam and Ro 15-1788. The compound **6d** was docked to see the effect of ligand charge as compared to **5c.** This pair of compounds (**5c** and **6d)** was seen in very much the same position with the difference that only **5c** was hydrogen bonded to γ_2Thr142. Compound **10** was apparently too bulky for the CBR, since the vast majority of the AutoDock clusters fell far away from the CBR, and the very few remaining ones showed unrealistic ligand distortions. Compound **11** was found to be too small to fill the CBR and therefore unable to make contact with α_1His101.

Accounting for the chiral selectivity, point mutation and photoaffinity labelling data as well as for the refined pharmacophore model for flavone derivatives binding, the $GABA_A$ receptor homology model can be used for predictive structural chemistry of novel CBR ligands. Comparison of the results obtained by in vitro and *in silico* affinity screens suggests that hydrogen bonding to γ_2Thr142 and nonpolar interaction with α_1His101 are essential for high affinity and $K_{i,PK}/K_{i,FLU}$ selectivity. Since the CBR is known to be dominantly hydrophobic [25,44], the small number of polar interactions found is not surprising. However, those present play an essential role in transducing the CBR conformational changes to the ion channel, and specifically γ_2Thr142 was suggested to play such a role [45,46]. It is to note, however, that PBR selective ligands, including PK 11195 and Ro 5-4864 as well as 4,1-benzothiazepine (CGP-37157), a specific inhibitor of the mitochondrial Na^+-Ca^{2+} exchanger, also successfully docked into the CBR site. Since the binding of the PBR-selective compounds to the CBR can be expected negligible, these findings indicate that docking results should be interpreted with care: other lines of evidence, such as point mutation and photoaffinity labelling data are to be matching.

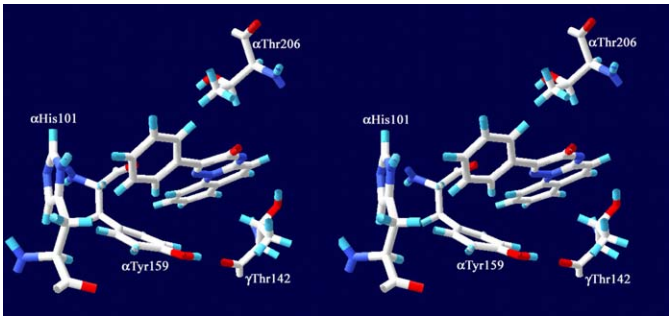


Fig. 2. Stereo view of the position of **1d** docked into the CBR binding site of the ErnstDC model relative to the most important residues for ligand binding.

5. Conclusions

Predictive structural chemistry was made possible by application of a homology model of the $GABA_A$ receptor extracellular domain confirming that hydrogen bonding to γ_2Thr142 and hydrophobic interaction with α_1His101 are essential for high CBR affinity and $K_{i,PK}/K_{i,FLU}$ selectivity. Structurally related sets of 33 condensed triazine derivatives, including 19 new compounds, were synthesised and screened for CBR and PBR affinity in vitro. New derivatives were characterised by a $K_{i,FLU}$ value of about a 40 nM for the triazino-quinoline derivative (**1 g**) as the lowest and $K_{i,PK}/K_{i,FLU}$ selectivity values ranging from >1300 to 2. By comparison, the best compound among the structurally related triazinophtalazines (Fig. 1.) had a K_i value of 220 nM [7]. Of particular interest may be the fact that low affinity may not exclude in vivo effectiveness. For example, some triazinophtalazines showed partial inhibition of conditioned avoidance and did not antagonised leptazol seizures [7]. Despite low affinity for CBR (IC_{50} values 4-8 μM), SR 95195 (Fig. 1.) showed inverse agonist properties in vivo [7]. Moreover, chrysin (Fig. 1.) that possesses low affinity for both CBR (3 μM) and PBR (13 μM) showed flumazenil-sensitive anticonvulsant effect in vivo [7]. Functional studies are required to explore the agonist (partial, inverse)/antagonist character of the most potent new triazino-quinoline derivative described.

6. Experimental protocols

6.1. Chemistry

Melting points are uncorrected. IR spectra were taken on a Thermo Nicolet Avatar 320 spectrophotometer. NMR spectroscopy measurements were carried out on a Varian Unity Inova (200 and 400 MHz) instrument. Chemical shifts are given relative to TMS=0.00 ppm. Elemental analyses (C, H, N) were carried out by Fisons Instrument (EA 1108 CHNS) automatic micro-analyser. The analytical results for the elements C, H, N were within ±0.4% of the theoretical values. For thin layer chromatography (TLC) analyses Silica gel 60 F_{254} sheets were used.

The following derivatives were prepared according to our previously published synthetic procedures: 2-iminoquinolin-1 (2H)-amine (**i**) [21], 3-methylthio-2-phenyl- [1,2,4]triazino [2,3-*a*]quinolinium perchlorate (**ii**)21, 1-iminoisoquinolin-2 (1H)-amine (**iii**) [21], 2-methoxy-3-phenyl- [1,2,4]triazino [3,2-*a*]isoquinolinium perchlorate (**iv**) [21], 1,2-diamino-pyridinium salt (**v**) [21], 3*H*- [1,2,4]triazino[2,3-*a*]quinolin-3-one (**1a**) [21], 2-methyl-3*H*- [1,2,4]triazino[2,3-*a*]quinolin-3-one (**1b**) [21], 2-phenyl-3*H*- [1,2,4]triazino[2,3-*a*]quinolin-3-one (**1d**) [21], 3-oxo-4-methyl-2-phenyl-3,4-dihydro [1,2,4]triazino[2,3-a]quinolin-11-ium tetrafluoroborate (**3b**) [21], 2-phenyl-3*H*- [1,2,4]triazino[2,3-*a*]quinoline-3-thione (**4**) [21], 2-phenyl-N'-[(3Z)-2-phenyl-3H- [1,2,4]triazino[2,3-*a*]quinolin-3-ylidene]acetohydrazide (**5b**) [21], 3-morpholin-4-yl-2-phenyl [1,2,4]triazino[2,3-*a*]quinolin-11-ium perchlorate (**6b**) [21], 3-

[(2-hydroxyethyl)amino]-2-phenyl [1,2,4]triazino[2,3-*a*]quinolin-11-ium ethanesulphonate (**6c**) [21], 3-benzyl-13-phenyl [1,2,4]triazolo[4′,3′:4,5] [1,2,4]triazino[2,3-*a*]quinolin-11-ium perchlorate (**7**)21, 2*H*- [1,2,4]triazino[3,2-*a*]isoquinolin-2-one (**8**) [22,47], 1-benzyl-4-phenyl [1,2,4]triazolo[4′,3′:4,5] [1,2,4] triazino[3,2-*a*]isoquinolin-6-ium perchlorate (**10**) [21,47], **ii** [21,47], **iv** [21], 3-(2-oxo-2-phenylethyl)-2H-pyrido[1,2-*b*] [1,2,4]triazin-2-one (**11**) [22], 2-(4-chlorophenyl)pyrido[1,2-*b*] [1,2,4]triazin-5-ium ethanesulphonate (**12**) [44], 3-(4-chlorophenyl)-2H-pyrido[1,2-*b*] [1,2,4]triazin-2-ol (**13**) [48], and 2-hydroxi-3-phenyl-1,2-dihydropyrido[1,2-*b*] [1,2,4]triazin-5-ium perchlorate (**14**) [48].

*6.1.1. General method for preparation of 2-substituted 3H-[1,2,4]triazino[2,3-a]quinolin-3-one (**1e-g**)*

To a suspension of 2-iminoquinolin-1(2H)-amine (**i**, 0.8 g, 5 mmol) in acetonitrile (5 mL) 6 mmol (1.2 equivalent) of the appropriate ethyl oxo-acetates was added dropwise with stirring. A crystalline precipitate was formed which after 4 h of stirring was filtered off. The product was recrystallised from acetonitrile.

*2-(4-chlorophenyl)-3H- [1,2,4]triazino[2,3-a]quinolin-3-one (**1e**)*

mp 286-288 °C (65%). IR (KBr): 3072 (CH); 1653, 1608, 1518, 1462 cm^{-1}. ^1H NMR (200 MHz; $CDCl_3$+TFA): 8.89 (d, 1H, *J*=8.0 Hz, H-10); 8.75 (d, 1H, *J*=8.6 Hz, H-6); 8.36, 7.70 (m, 4H, H-C_6H_4); 8.22 (t, 1H, *J*=7.8 Hz, H-9); 8.19 (d, 1H, *J*=7.8 Hz, H-7); 7.98 (t, 1H, *J*=7.8 Hz, H-8); 7.77 (d, 1H, *J*=8.6 Hz, H-5). Anal. ($C_{17}H_{10}ClN_3O$) C, H, N.

*2-(4-fluorophenyl)-3H- [1,2,4]triazino[2,3-a]quinolin-3-one (**1f**)*

mp 285-287 °C (58%). IR (KBr): 3072 (CH); 1653, 1605, 1519, 1510, 1462, 1447 cm^{-1}. ^1H NMR (200 MHz; $CDCl_3$ +TFA): 8.92 (d, 1H, *J*=8.6 Hz, H-10); 8.76 (d, 1H, *J*=9.2 Hz H-6); 8.48, 7.80 (m, 4H, H-C_6H_4); 8.21 (dd, 1H, *J*=6.8, 8.6 Hz, H-9); 8.20(d, 1H, *J*=8.8 Hz, H-7); 7.99 (dd, 1H, *J*=8.8, 6.8 Hz, H-8); 7.35 (d, 1H, *J*=9.2 Hz, H-5). Anal. ($C_{17}H_{10}FN_3O$) C, H, N.

*2-(4-methylphenyl)-3H- [1,2,4]triazino[2,3-a]quinolin-3-one (**1 g**)*

mp 226-227 °C (55%). IR (KBr): 3042, 1653, 1611, 1545, 1518, 1464 cm^{-1}. ^1H NMR (400 MHz; $CDCl_3$): 8.62 (d, 1H, *J*=8.6 Hz, H-10); 8.36, 7.32 (m, 4H, H-C_6H_4); 7.94 (d, 1H, *J*=9.4 Hz, H-6); 7.79 (dd, 1H, *J*=7.8, 7.0 Hz, H-9); 7.77 (d, 1H, *J*=7.8 Hz, H-7); 7.56 (dd, 1H, *J*=8.6, 7.0 Hz, H-8); 7.24 (d, 1H, *J*=9.4 Hz, H-5); 2.42 (s, 3H, H-CH_3). ^{13}C NMR: 161.0 (C-3); 150.7 (C-4a); 150.5 (C-2); 142.1 (C-4'); 138.0 (C-6); 137.5 (C-10a); 132.0 (C-9); 130.0 (C-3',5'); 129.7 (C-1'); 129.3(C-2',6'); 128.9 (C-7); 126.9 (C-8); 122.3 (C-5); 116.7 (C-10); 21.6 (C-CH_3). Anal. ($C_{18}H_{13}N_3O$) C, H, N.

*Ethyl 3-oxo-3H- [1,2,4]triazino[2,3-a]quinoline-2-carboxylate (**1c**)*

mp 201-203 °C (55%). IR (KBr): 3070, 1742, 1662, 1618, 1520, 1465 cm^{-1}. ^1H NMR (200 MHz; DMSO-d_6): 8.34 (d, 1H, *J*=8.8 Hz, H-10); 8.33 (d, 1H, *J*=9.4, H-6); 8.04 (d, 1H, *J*=7.8, H-7); 7.88 (dd, 1H, *J*=7.8, 7.4, H-8); 7.68 (dd, 1H, *J*=8.8, 7.4, H-9); 7.24 (d, 1H, *J*=9.4, H-5); 4.46 (q, 2H, H-CH_2); 1.37 (t, 3H, H-CH_3). ^{13}C NMR: 161.8; 157.6; 151.3; 139.4; 136.6; 132.0; 129.2; 127.0; 122.9; 121.9; 115.6; 62.4; 13.9. Anal. ($C_{14}H_{11}N_3O_3$) C, H, N.

*6.1.2. General method for preparation of 2-substituted 3-oxo-3,4-dihydro [1,2,4]triazino[2,3-a]quinolin-11-ium salts (**2a-d**)*

To a suspension of 3*H*- [1,2,4]triazino[2,3-*a*]quinolin-3-one derivatives (**1a**, **1d**, **1 g**, 1 mmol) in acetonitrile (2 mL) 1.5 mmol (1.5 equivalent) of the appropriate acid (ethanesulphonic acid, tetrafluoroboric acid, perchloric acid) was added dropwise with stirring and a clear solution was formed. A white crystalline product was precipitated after adding diethylether (5 mL) to the solution which was filtered off. The product was recrystallised from acetonitrile-diethylether mixture.

*3-oxo-2-phenyl-3,4-dihydro [1,2,4]triazino[2,3-a]quinolin-11-ium ethanesulphonate (**2a**)*

mp 163-168 °C (75%). IR (KBr): 3054, 3006, 1714, 1618, 1543, 1338, 1209, 1146 cm^{-1}. ^1H NMR (200 MHz; DMSO-d_6) : 8.64 (d, 1H, *J*=8.6 Hz, H-10); 8.38 (d, 1H, *J*=9.4 Hz, H-5); 8.3, 7.6 (m, 5H, H-phenyl); 8.09 (d, 1H, *J*=7.8 Hz, H-7); 7.94 (dd, 1H, *J*=8.6, 7.2 Hz, H-9); 7.70 (dd, 1H, *J*=7.2, 7.4 Hz, H-8); 7.34 (d, 1H, *J*=9.4 Hz, H-6); 2.46 (q, 2H, H-CH_2-$EtSO_3$); 1.09 (t, 3H, H-CH_3-$EtSO_3$). Anal. ($C_{19}H_{17}N_3O_4S$) C, H, N.

*3-oxo-2-phenyl-3,4-dihydro [1,2,4]triazino[2,3-a]quinolin-11-ium tetrafluoroborate (**2b**)*

mp 203-206 °C (78%). IR (KBr): 3588, 3089, 1727, 1622, 1530, 1083 cm^{-1}. The proton and carbon signals are identical with **2a** in the NMR spectra. Anal. ($C_{17}H_{12}BF_4N_3O$) C, H, N.

*3-oxo-2-phenyl-3,4-dihydro [1,2,4]triazino[2,3-a]quinolin-11-ium perchlorate (**2c**)*

mp 287-294 °C (82%). IR (KBr): 3080, 1732, 1662, 1621, 1545, 1108 cm^{-1}. The proton and carbon signals are identical with **2a** in the NMR spectra. Anal. ($C_{17}H_{12}ClN_3O_5$) C, H, N.

*3-oxo-2-(4-methylphenyl)-3,4-dihydro [1,2,4]triazino[2,3-a] quinolin-11-ium tetrafluoroborate (**2d**)*

mp 232-234 °C (72%). IR (KBr): 3576, 3096, 1726, 1625, 1526, 1341, 1084 cm^{-1}. ^1H NMR (200 MHz; DMSO-d_6): 8.69 (d, 1H, *J*=8.6 Hz, H-10); 8.48 (d, 1H, *J*=9.6 Hz, H-6); 8.33, 7.37 (m, 4H, H-C_6H_4); 8.14 (dd, 1H, *J*=7.8, 1.5 Hz, H-7); 8.0 (dd, 1H, *J*=7.4, 8.6 Hz, H-8); 7.75 (dd, 1H, *J*=7.8, 7.4 Hz, H-9); 7.41 (d, 1H, *J*=9.6, H-5); 2.44 (s, 3H, H-CH_3). ^{13}C NMR: 158.0; 149.1; 141.7; 140.0; 136.5; 132.6; 129.5; 129.4; 129.0; 127.2; 123.2; 119.5; 116.2; 21.1 (C-CH_3). Anal. ($C_{18}H_{14}BF_4N_3O$) C, H, N.

*6.1.3. General method for preparation of 2-substituted 3-oxo-4-methyl-3,4-dihydro [1,2,4]triazino[2,3-a]quinolin-11-ium salts (**3a, 3c**)*

To a suspension of 3*H*- [1,2,4]triazino[2,3-*a*]quinolin-3-one derivatives (**1d**, **1 g**, 1 mmol) in acetonitrile (2 mL) dimethyl sulphate (0.5 mL) was added, and the mixture was refluxed for 5 h. The reaction mixture was evaporated, iced water and tetrafluoroboric acid (0.5 mL) was added to the residue, the precipitated white crystals were filtered off and recrystallised from acetonitrile-diethylether mixture.

*3-oxo-4-methyl-2-phenyl-3,4-dihydro [1,2,4]triazino[2,3-a] quinolin-11-ium ethanesulphonate (**3a**)*

mp 198-200 °C (62%). IR (KBr): 3035, 1709, 1617, 1532, 1446, 1187 cm^{-1}. ^1H NMR (200 MHz; DMSO-d_6): 9.26 (d, 1H, *J*=9.6, H-6); 9.00 (d, 1H, *J*=8.6 Hz, H-10); 8.45 (d, 1H, *J*=8.2 Hz, H-7); 8.35, 7.74 (m, 5H, H-C_6H_5); 8.40 (d, 1H, *J*=9.6 Hz, H-5); 8.29 (dd, 1H, *J*=8.0, 8.6 Hz, H-9); 8.05 (dd, 1H, *J*=8.2, 8.0 Hz, H-8); 3.95 (s, 3H, H-N-CH_3); 2.32 (q, 2H, H-CH_2-$EtSO_3$); 1.01 (t, 3H, H-CH_3-$EtSO_3$). ^{13}C NMR: 152.6; 150.3; 146.4; 146.3; 136.1; 135.6; 133.0; 130.2; 130.0; 129.9; 129.3; 128.9; 124.0; 117.5; 112.3; 45.0; 31.6; 9.9. Anal. ($C_{20}H_{19}N_3O_4S$) C, H, N.

*3-oxo-4-methyl-2-(4-methylphenyl)-3,4-dihydro [1,2,4]triazino[2,3-a]quinolin-11-ium tetrafluoroborate (**3c**)*

mp 298-302 °C (75%). IR (KBr): 3576, 3096, 1726, 1625, 1526, 1341, 1084 cm^{-1}. ^1H NMR (200 MHz; DMSO-d_6): 9.22 (d, 1H, *J*=9.6 Hz, H-5); 9.04 (d, 1H, *J*=8.4 Hz, H-10); 8.45 (d, 1H, *J*=8.2 Hz, H-7); 8.33, 7.53 (m, 4H, H-C_6H_4); 8.31 (d, 1H, *J*=9.6 Hz, H-6); 8.29 (dd, 1H, *J*=8.2, 8.4 Hz, H-9); 8.04 (t, 1H, *J*=8.2 Hz, H-8); 3.91 (s, 3H, H-N-CH_3); 2.49 (s, 3H, H-CH_3). ^{13}C NMR: 152.3; 150.4; 146.3; 146.2; 143.7; 136.2; 135.6; 130.2; 130.0; 129.6; 129.4; 127.2; 124.1; 117.6; 112.2; 31.6; 21.3. Anal. ($C_{19}H_{16}BF_4N_3O$) C, H, N.

*benzyl[(3Z)-2-phenyl-3H- [1,2,4]triazino[2,3-a]quinolin-3-ylidene]amine (**5a**)*

To a suspension of 3-methylthio-2-phenyl- [1,2,4]triazino [2,3-*a*]quinolinium perchlorate (**ii**, 0.8 g, 2 mmol) in acetonitrile (5 mL) benzylamine (0.43 g, 4 mmol) was added dropwise. A deep red solution was formed first from which yellow crystals separated, the mixture was stirred for additional 2 h, and the precipitated product was filtered off to give 0.57 g (80%), mp 106-108 °C. IR (KBr): 3025, 2876, 1629, 1589, 1524, 1325 cm^{-1}. ^1H NMR (200 MHz; DMSO-d_6): 8.31 (m, 2H, H-6, 10); 8.05 (d, 1H, *J*=7.0 Hz, H-7); 7.77 (t, 1H, *J*=7.0 Hz, H-8); 7.64-7.20 (m, 12H, H-9, 5, C_6H_5); 4.78 (s, 2H, H-CH_2). Anal. ($C_{24}H_{18}N_4$) C, H, N.

*2-phenyl-3H- [1,2,4]triazino[2,3-a]quinolin-3-imine (**5c**)*

To a suspension of 3-methylthio-2-phenyl- [1,2,4]triazino [2,3-*a*]quinolinium perchlorate (**ii**, 0.8 g, 2 mmol) in acetonitrile (5 mL) ammonium hydroxide (1 mL) was added dropwise. A deep red solution was formed first from which yellow crystals separated, the mixture was stirred for additional 2 h, and the precipitated product was filtered off to give 0.40 g (75%) mp 200-203 °C. IR (KBr): 3265, 3056, 1657, 1609, 1519, 1464 cm^{-1}. ^1H NMR (200 MHz; DMSO-d_6): 10.0 (br, 1H, NH); 8.73 (d, 1H, *J*=9.2 Hz, H-6); 8.67 (d, 1H, *J*=8.6 Hz, H-10); 8.26 (d, 1H, *J*=7.9 Hz, H-7); 8.05 (t, 1H, *J*=7.4 Hz, H-8); 7.95-7.66 (m, 7H, H-9, 5, C_6H_5). ^{13}C NMR: 153.8; 148.5; 146.9; 141.8; 136.3; 133.2; 131.8; 130.2; 129.4; 129.0; 128.5; 124.1; 120.8; 116.5. Anal. ($C_{17}H_{12}N_4$) C, H, N.

*3-{[2-(dimethylamino)ethyl]amino}-2-phenyl [1,2,4]triazino [2,3-a]quinolin-11-ium bromide (**6a**)*

To a suspension of 3-methylthio-2-phenyl- [1,2,4]triazino [2,3-*a*]quinolinium perchlorate (**ii**, 0.8 g, 2 mmol) in acetonitrile (5 mL) 2-(dimethylamino)ethylamine (0.35 g, 4 mmol) was added dropwise. A deep red solution was formed first from which yellow crystals separated, the mixture was stirred for additional 2 h, and the precipitated product was filtered off. This solid product was suspended in acetonitrile (2 mL) and hydrobromide (0.2 mL, 48% in water) was added and upon heating a clear solution was formed which was cooled down and the precipitated bromide salt was filtered off to give 0.42 g (65%) mp 219-220 °C. IR (KBr): 3462, 3385, 2944, 1597, 1520, 1456 cm^{-1}. ^1H NMR (200 MHz; $CDCl_3$+ TFA): 8.85 (d, 1H, *J*=8.5 Hz, H-6); 8.58 (d, 1H, *J*=8.2 Hz, H-10); 8.1 (m, 3H, H-7,8,9); 7.9-7.6 (m, 6H, H-phenyl, H-5); 4.41 (q, 2H, H-CH_2); 3.69 (q, 2H, H-CH_2); 3.05, 2.98 H-N(CH_3)$_2$. Anal. ($C_{21}H_{22}BrN_5$) C, H, N.

*3-amino-2-phenyl [1,2,4]triazino[2,3-a]quinolin-11-ium perchlorate (**6d**)*

To a suspension of 3-methylthio-2-phenyl- [1,2,4]triazino [2,3-*a*]quinolinium perchlorate (**ii**, 0.8 g, 2 mmol) in acetonitrile (5 mL) ammonium hydroxide (0.5 mL) was added dropwise. A yellow solution was formed first from which pale yellow crystals separated, the mixture was stirred for additional 2 h, ethyl acetate (5 mL) was added to this mixture and the precipitated product was filtered off to give 0.50 g (68%) mp 221-224 °C. IR (KBr): 3436, 3305, 3201, 1646, 1615, 1492, 1453, 1099 cm^{-1}. ^1H NMR (200 MHz; $CDCl_3$+ TFA): 8.95 (d, 1H, *J*=8.4 Hz, H-6); 8.68 (d, 1H, *J*=8.2 Hz, H-10); 8.15 (m, 3H, H-7,8,9); 7.9-7.4 (m, 6H, H-phenyl, H-5). Anal. ($C_{17}H_{13}ClN_4O_4$) C, H, N.

*2-amino-3-phenyl [1,2,4]triazino[3,2-a]isoquinolin-5-ium ethanesulphonate (**9a**)*

To a suspension of 2-methoxy-3-phenyl- [1,2,4]triazino[3,2-*a*]isoquinolinium perchlorate (**iv**, 0.78 g, 2 mmol) in acetonitrile (5 mL) ammonium hydroxide (0.5 mL) was added dropwise. A yellow solution was formed first from which pale yellow crystals separated, the mixture was stirred for additional 2 h, ethyl acetate (5 mL) was added to this mixture and the precipitated product was filtered off. This solid product was suspended in acetonitrile (2 mL) and ethanesulphonic acid (0.1 mL) was added and upon heating a clear solution was formed which was cooled down and the precipitated salt was filtered off to give 0.50 g (68%) mp 217-220 °C. IR (KBr): 3432, 3065, 2925, 1639, 1568, 1474, 1458, 1221 cm^{-1}. ^1H NMR (200 MHz; DMSO-d_6+ $CDCl_3$): 9.5 (br, NH); 8.88 (d, 1H, *J*=8.1 Hz, H-11); 8.50 (d, 1H, *J*=7.4 Hz, H-6); 8.08 (dd, 1H, *J*=7.9, 6.4 Hz, H-9); 7.95 (dd, 1H, *J*=8.1, 6.4 Hz, H-10); 7.82 (dd, 1H, *J*=7.9 Hz, H-8); 7.65 (d, 1H, *J*=7.4 Hz, H-7); 8.1, 7.60 (m, 5H, H-phenyl); 2.46 (q, 2H, H- CH_2); 1.10 (t, 3H, H-CH_3). ^{13}C NMR: 153.3; 148.1; 135.0; 134.5; 131.6; 129.8; 129.7; 129.1; 128.7; 127.5; 126.4; 123.4; 117.3; 45.1; 9.8. Anal. ($C_{19}H_{18}N_4$ O_3S) C, H, N.

*2-{[3-(dimethylamino)propyl]amino}-3-phenyl [1,2,4]triazino[3,2-a]isoquinolin-5-ium ethanesulphonate (**9b**)*

To a suspension of 2-methoxy-3-phenyl- [1,2,4]triazino[3,2-*a*]isoquinolinium perchlorate (**iv**, 0.78 g, 2 mmol) in acetonitrile (5 mL) 3-(dimethylamino)propylamine (0.41 g, 4 mmol) was added dropwise. A yellow solution was formed first from which pale yellow crystals separated, the mixture was stirred for additional 2 h, ethyl acetate (5 mL) was added to this mix-

ture and the precipitated product was filtered off. This solid product was suspended in acetonitrile (2 mL) and ethanesulphonic acid (0.1 mL) was added and upon heating a clear solution was formed which was cooled down and the precipitated salt was filtered off to give to give 0.55 g (55%) mp 169-171 °C. IR (KBr): 3449, 2966, 1640, 1599, 1483, 1467, 1185 cm^{-1}. ^1H NMR (200 MHz; DMSO-d_6+ $CDCl_3$): 9.5 (br, NH); 9.08 (d, 1H, *J*=8.1 Hz, H-11); 8.56 (d, 1H, *J*=7.4 Hz, H-6); 8.14 (dd, 1H, *J*=7.9, 6.4 Hz, H-9); 8.01 (dd, 1H, *J*=8.1, 6.4 Hz, H-10); 7.84 (dd, 1H, *J*=7.9 Hz, H-8); 7.67 (d, 1H, *J*=7.4 Hz, H-7); 8.1, 7.60 (m, 5H, H-phenyl); 3.91 (t, 2H, H-CH_2); 3.41(s, 3H, N-CH_3); 3.31 (t, 2H, H- CH_2); 2.83 (s, 3H, N-CH_3); 2.50 (q, 2H, H- CH_2); 2.23 (t, 2H, H- CH_2); 1.10 (t, 3H, H-CH_3). ^{13}C NMR: 151.4; 148.8; 135.0; 134.6; 131.5; 129.9; 129.3; 129.1; 129.0; 127.4; 126.9; 123.6; 117.4; 54.4; 45.0; 42.2; 22.8; 9.6. Anal. ($C_{24}H_{29}N_5O_3S$) C, H, N.

*2-amino [1,2,4]triazino[3,2-a]isoquinolin-5-ium bromide hydrate (***9c***)*

To a suspension of 2-methoxy- [1,2,4]triazino[3,2-*a*]isoquinolinium perchlorate (**iv**, 0.62 g, 2 mmol) in acetonitrile (5 mL) ammonium hydroxide (0.5 mL) was added dropwise. A yellow solution was formed first from which pale yellow crystals separated, the mixture was stirred for additional 2 h, ethyl acetate (5 mL) was added to this mixture and the precipitated product was filtered off. This solid product was suspended in acetonitrile (2 mL) and hydrobromide (0.2 mL, 48% in water) was added and upon heating a clear solution was formed which was cooled down and the precipitated bromide salt was filtered off to give 0.36 g (62%) mp 269-270 °C. IR (KBr): 3416, 3354, 3031, 1677, 1606, 1485, 1447 cm^{-1}. ^1H NMR (200 MHz; $CDCl_3$+TFA): 9.03 (d, 1H, *J*=8.0 Hz, H-11); 8.82 (s, 1H, H-3); 8.30 (d, 1H, *J*=7.4 Hz, H-6); 8.14 (dd, 1H, *J*=7.9, 7.4 Hz, H-9); 8.12 (d, 1H, *J*=7.9 Hz, H-8); 7.98 (dd, 1H, *J*=8.0, 7.4 Hz, H-10); 7.73 (d, 1H, *J*=7.4 Hz, H-7). Anal. ($C_{11}H_{11}BrN_4O$) C, H, N.

*2-(benzylamino)-3-[(benzylamino)carbonyl] [1,2,4]triazino [3,2-a]isoquinolin-5-ium ethanesulphonate (***9d***)*

To a suspension of 3-(ethoxycarbonyl)-2-methoxy- [1,2,4] triazino[3,2-*a*]isoquinolin-5-ium perchlorate (**iv**, 0.76 g, 2 mmol) in acetonitrile (5 mL) benzylamine (1.07 g, 10 mmol) was added dropwise. A yellow solution was formed first from which pale yellow crystals separated, the mixture was stirred for additional 6 h, ethyl acetate (5 mL) was added to this mixture and the precipitated product was filtered off. This solid product was suspended in acetonitrile (2 mL) and ethanesulphonic acid (0.1 mL) was added and upon heating a clear solution was formed which was cooled down and the precipitated salt was filtered off to give to give 0.54 g (50%) mp 203-206 °C. IR (KBr): 3236, 3055, 2991, 1678, 1608, 1486, 1183 cm^{-1}. ^1H NMR (200 MHz; DMSO-d_6+ $CDCl_3$): 10.95, 10.25 (br, NH); 8.96 (d, 1H, *J*=8.1 Hz, H-11); 8.52 (d, 1H, *J*=7.3 Hz, H-6); 8.16 (dd, 1H, *J*=8.1, 6.4 Hz, H-10); 8.01 (dd, 1H, *J*=7.8, 6.4 Hz, H-9); 7.59 (d, 1H, *J*=7.8 Hz, H-8); 7.57 (d, 1H, *J*=8.1 Hz, H-7); 7.5-7.30 (m, 10H, H-phenyl); 5.10 (s, 2H, H-CH_2); 4.61(s, 2H, H-CH_2); 2.45 (q, 2H, H- CH_2); 1.12 (t, 3H, H-CH_3). ^{13}C NMR: 160.7; 150.5; 148.5; 137.8; 136.4; 135.6; 135.3; 134.8; 131.4; 130.1; 128.4; 128.2; 128.0; 127.6; 127.4; 127.2; 127.0; 123.2; 117.9; 45.0; 44.9; 42.7; 9.8. Anal. ($C_{29}H_{31}N_5O_4S$) C, H, N.

6.2. In Vitro Pharmacology

3-6 weeks old male Wistar rats purchased from Toxicoop (Budapest, Hungary) were kept and used in accordance with the European Council Directive of 24 November 1986 (86/609/EEC) and with the Hungarian Animal Act, 1998 and associated guidelines. Compounds used in the experiments: tris(hydroxymethyl)aminomethane (TRIS), NaCl, saccharose, (Reanal: Budapest, Hungary); HiSafe 3 scintillation mixture (LKB-Wallac: Bromma, Sweden); diazepam (Sigma: Budapest, Hungary); PK 11195, Ro 15-1788 (Tocris: Bristol, UK); [^{3}H]-Flunitrazepam (77 Ci/mmol) (Amersham: Little Chalfont, UK); [^{3}H]-PK 11195 (83.5 Ci/mmol); and spectroscopic grade dimethyl sulphoxide (DMSO, Merck: Budapest, Hungary). Buffers had the following compositions: A – 50 mM Tris HCl adjusted to pH 7.1 at 4 °C with HCl; B – 5 mM TRIS adjusted to pH 7.4 with HCl; C – 50 mM TRIS adjusted to pH 7.4 with HCl.

In the displacement experiments, the concentration of test compounds varied from 1 nM to 100 μM. Stock solutions of tested compounds were made in DMSO, the final concentration of DMSO in the assay being 0.5%. Duplicate samples (2 x 500 μL) were filtered through micro-porous glass fibre filters (Whatman GF/B) pre-soaked with 5 ml ice-cold buffer, and washed 3 times with 5 mL buffer. The radioactivity of the filters was counted in Optiphase HiSafe3 LKB scintillation mixture with an efficiency of 30%.

6.2.1. [^{3}H]-Flunitrazepam binding to synaptic membranes isolated from the rat brain cortex

Synaptic membrane fractions were prepared as described by Braestrup and Squires [6]. Cortices were dissected on ice, homogenised in 10 volumes of ice-cold 0.32 M sucrose solution with Potter-Elvehjem glass-Teflon homogeniser. The homogenate was pelleted at 1 500 *g* for 10 min, and the supernatant was stored. The pellet was re-suspended in the same volume of 0.32 M sucrose. The combined supernatants were centrifuged at 9 500 *g* for 20 min. The pellet was re-suspended in 20 mL buffer A to give a protein concentration of 0.6-0.7 mg/ml [49] and used in [3H]-flunitrazepam binding experiments. Binding experiments were performed as described by Kardos et al. [50] with minor modifications. Aliquots (600 μL) of synaptosomal membrane homogenate in buffer A were incubated with 600 μL of buffer A solution containing [^{3}H]-flunitrazepam (final concentration 2 nM) in the absence or (to define non-specific binding) the presence of 10 μM diazepam for 60 min at 4.0±0.1 °C. Under the conditions, the non-specific to total ratio for [^{3}H]-flunitrazepam binding was 0.10.

6.2.2. [^{3}H]-PK 11195 binding to membranes isolated from the rat forebrain

Membrane homogenates were prepared and [^{3}H]-PK 11195 binding experiments were performed as described by Maksay

[25]. Shortly, forebrains were dissected on ice, homogenised in 10 volumes of buffer B at 20 000 rpm for 5 s with an Ultra-Turrax homogeniser. The suspension was centrifuged at 40 000 *g* for 20 minutes at 4 °C. The pellet was re-suspended in 20 mL of buffer C and used for [^{3}H]-PK 11195 binding experiments. Aliquots (600 μL) of synaptosomal membrane homogenate in buffer C were incubated with the same volume of buffer C solution containing [^{3}H]-PK 11195 (final concentration 0.6 nM) for 90 min at 4.0±0.1 °C. Non-specific binding was determined in the presence of 50 μM PK 11195. The non-specific to total ratio for [^{3}H]-PK 11195 binding was 0.13.

6.2.3. Data evaluation

IC_{50} values determined from 2-4 displacement experiments were given as mean±SEM. The $K_{i,FLU}$ and $K_{i,PK}$ values of the tested compounds were calculated using the Cheng–Prussof equation [51]: $K_i=IC_{50}/1+c^*/K_d$, where c* refers to the concentration of the radiolabel and K_d for the equilibrium dissociation constant of the radioligand, using the K_d values of [^{3}H]-Flunitrazepam [52] and [3H]-PK 11195 binding [53], 0.86 nM and 1.41 nM, respectively.

6.3. Molecular modelling

The model [20] of the GABAA receptor was downloaded from Margot Ernst's web page [54]. The model contains parts of the extracellular domains of the α_1, β_2 and γ_2 subunits truncated N-terminally at Ile17, Val16 and Ile30, respectively, and C-terminally at the end of the extracellular domain (Gly223, Gly219 and Gly234, respectively). From the pentameric PDB model the α_1 (from Ile17 to Gly233) and γ_2 (from Ile30 to Gly234) peptide chains embracing the benzodiazepine binding site were cut out. This dimeric construct is referred to as ErnstDC and its energy-minimised version as the apo dimer structure. All molecular mechanics calculations were done by the SYBYL molecular modelling software (version 6.6; Tripos Associated Ltd.: St. Louis, MO, USA), as described previously [55] with the following modifications. The Tripos force field [56] was used throughout the calculations for its simplicity, robustness and considerably faster execution times as compared to other, more sophisticated force fields, and because of the inherent uncertainty of side-chain positions in the ErnstDC model. Energy minimisation was done with Gasteiger-Hückel charges using the Powell method for 10000 steps or until the gradient was lower than 0.01 kcal mol^{-1} Å^{-1}. The structures of the compounds studied were constructed using the SYBYL package.

For flunitrazepam and Ro 15-1788 both the P and M conformers were built. After simulated annealing (10 cycles, heat to 700 K for 1000 fs, anneal to 200 K for 1000 fs), the ligands were energetically minimised. The initial docking position of the ligand in the apo dimer was determined by the AutoDock 3.0 program [57] by using the default box (22.5x22.5x22.5 Å) and grid (0.375 Å) parameters, 100 runs and an rms-tolerance of 1 Å for the distinct conformational clusters. The ligand with the lowest energy of the most populated cluster was transferred to the apo dimer, and the complex energy-minimised in SYBYL allowing flexible geometry for both ligand and protein. The hydrophobic interactions were analysed and plotted by the LIGPLOT program [58].

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Supplementary material

The full co-ordinates of the compound **1d** docked into the ErnstDC structure can be downloaded from www.medichem.hu/ejmc/index.html in mol2 and pdb format.

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