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Interaction of clozapine and its nitrenium ion with rat D2 dopamine receptors: in vitro binding and computational study

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Abstract The interaction of diazepine analogues like clozapine or olanzapine with D2 receptor was greatly affected by a mixture of HRP/H_2O_2 known to induce the formation of nitrenium ion. Unlike diazepine derivatives, the oxidative mixture had low impact on the affinity of oxa- and thiazepine derivatives such as loxapine, clothiapine or JL13 for the D2 receptor. Molecular docking simulations revealed a huge difference between the mode of interaction of clozapine nitrenium ion and the parent drug. Electronic and geometric changes of the tricyclic ring system caused by the oxidation appeared to prevent the compound finding the correct binding mode and could therefore explain the difference observed in binding affinities.

Keywords Diazepine · Nitrenium · Oxidation · Dopamine D2 receptor · Homology modelling · Molecular docking

Abbreviations

GPCR	G protein-coupled receptor
ICL	Intracellular loop
ECL	Extracellular loop
MD	Molecular dynamics
HRP	Horseradish peroxidase
EDTA	Ethylene diamine tetraacetic acid

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Introduction

Due to its original antipsychotic profile and the presence of several side effects, clozapine has generated numerous theories to explain the biological basis for its advantages and to find a safer successor. The 5-HT_2/D2 pKi ratio hypothesis [1] led to the development of the second generation of antipsychotic drugs with the discovery of compounds like risperidone, olanzapine or quetiapine. Other works indicate that the most powerful predictor of atypicality for the current generation of atypical antipsychotics is fast dissociation from the D2 receptor and not a high affinity at other receptors such as 5-HT_2 or D4 receptors [2]. Nevertheless, so far, the precise mechanism of action of these compounds is not yet fully explained and remains a matter of debate.

Otherwise, the formation of nitrenium species following oxidation processes was proposed to explain the haematological toxicity of clozapine [3]. Indeed, nitrogen derivatives like clozapine or olanzapine (Fig. 1) are very sensitive to oxidation while oxygen or sulphur isosteres such as loxapine, clothiapine and JL13 (Fig. 1), possess a very low sensitivity to oxidation [4–6]. Despite an extensive effort to examine the impact of such oxidized compounds in different models supposed to be predictive of haematological toxicity to our knowledge no evaluation of such entities in terms of receptor interaction was reported.

In the present study, we have evaluated the affinity of different tricyclic derivatives for rat D2 receptors in normal and oxidative conditions. Then, we used a structure-based approach to understand the impact of oxidative conditions on the binding of these molecules on rat dopamine D2 receptors. Therefore, docking studies of clozapine and its nitrenium ion were performed on a homology model of D2

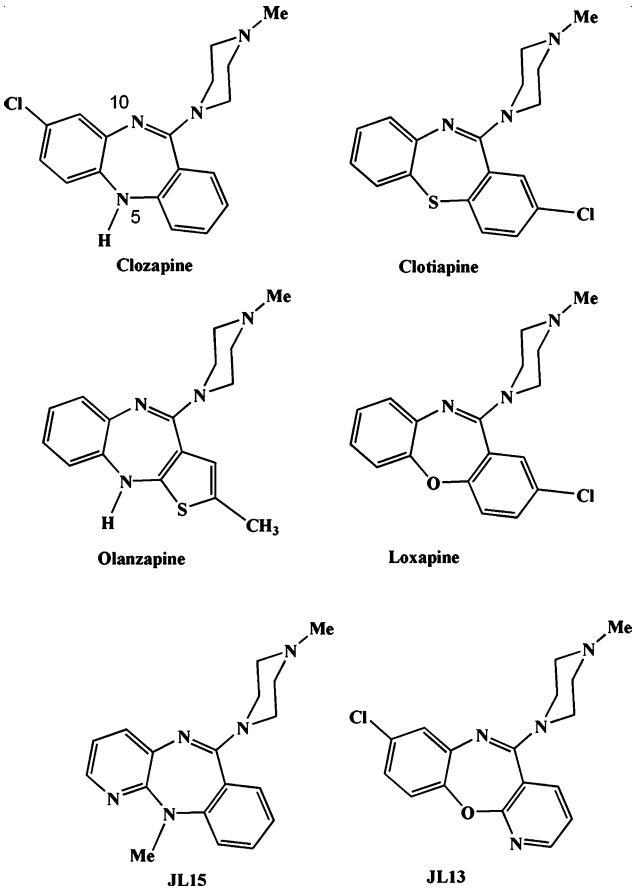

Fig. 1 Chemical structure of clozapine and related analogues

receptor to reveal possible difference in binding interactions.

Materials and methods

In vitro binding experiments

All procedures were carried out in accordance with guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were accepted by the Ethics Committee for Animal Use of the University of Liège.

Female Wistar rats weighted ~250 g were used. The brains were quickly removed after cerebral dislocation and dissected on ice to get striata. After weighting, tissues were homogenized in Tris 50 mM buffered at pH 7.7 with 4 N aqueous HCl and washed three times by centrifugation. The final pellet was dispersed in the appropriate volume of incubation buffer depending on the experimental conditions. Protein determination was made by using previously described procedure [7].

Normal incubation buffer contained Tris 50 mM, $MgCl_2$ 5 mM, and EDTA disodium salt 1 mM buffered at pH 7.4 with 4 N aqueous HCl while in oxidative conditions horseradish peroxidase (1.25 μg/tube) and H_2O_2 (50 μM) were added a few minutes before the addition of membrane preparation. These concentrations were similar to those used in previous studies. [^{3}H]-Spiperone (Perkin Elmer, 15 Ci/mmol) was used as radioligand and the non specific binding was determined in the presence of haloperidol (10 μM). Incubation temperature and time were 27 °C and 60 min, respectively. After this period of time, samples were filtered on Whatman GF/C fiber glass filters presoaked in 0.3% aqueous PEI (Sigma) and the filters were washed two times with Tris 50 mM buffered at pH 7.4 with 4 N aqueous HCl. Affinities are expressed as Ki after a classical transformation [8]. The oxidative mixture might have deleterious effects on the receptor structure but the Kd of spiperone in normal versus oxidation conditions were found to be very similar.

D2 receptor modelling

The receptor model was built by homology modelling using the SYBYL® 8.0 molecular modelling package (SYBYL 8.0, 2008, Tripos Inc., 1699 South Hanley Road, St. Louis, MO 63144-2913, USA). It consisted of several steps. First, the rat sequence of the D2 receptor extracted from the Universal Protein Resource (http://www.uniprot.org/; code entry P61169) [9] was aligned with the sequence of the human β2 adrenergic GPCR (code entry P07550) [10] by the use of the FUGUE™ sequence alignment module [11]. Great attention was paid to a correct alignment of the highly conserved residues of the GPCR superfamily according to Baldwin et al. [12]. The second step was the transfer of a set of constraints derived from the crystal structure of the human β2 adrenergic GPCR (PDB entry 2RH1) [13] to the corresponding residues of the sequence to be modelled. These constraints allowed the construction of the model backbone of the transmembrane α-helices using the CHORAL program included in the ORCHESTRAR™ protein structure modelling module [14]. As the T4-lysozyme replaced the intracellular loop ICL3, which was found to have no direct effect on ligand binding, ICL3 was omitted in the receptor modelling. Other loops were modeled by the CODA program of ORCHESTRAR™ [15]. CODA combines knowledge-based and ab initio approaches to predict the structurally variable regions of protein models. We then used the ANDANTE program of ORCHESTRAR™ that has the advantage of placing the side chains from a library of rotamers derived from high resolution crystal structures, and thus taking into account the environment of a residue to orient appropriately its side chain [16]. The conserved disulfide bond between the cysteine Cys 3.25 (Ballesteros-Weinstein nomenclature) [17] and the cysteine in the middle of

extracellular loop ECL2 was also created and was kept as a constraint in the model refinement.

The output structure was then iteratively energy minimized using the Powell method available in Maximin2 procedure [18] with the AMBER 7 FF99 force field [19] including the electrostatic term, a dielectric constant set to four and a nonbond distance cut-off of 12 Å. It is however necessary to preserve the geometry of the backbone to keep the tertiary structure of the alpha helices. We thus carried out this minimization in three stages. First, the whole backbone was constrained for 1,000 iterations to remove steric clashes of the side chains. Then the backbone of the helices was constrained while the loops were allowed to move for 2,000 iterations. A final 2,000 cycles was carried out on the whole protein.

Finally, PROCHECK software [20] was used to assess the stereochemical quality of the refined model. Loops were not taken into account as their more flexible nature renders them more prone to structural mistakes in a static single conformation snapshot such as the Ramachandran plot. This analysis resulted in high-quality parameters with a very good distribution of φ and Ψ angles. More than 98% of the residues were found in the most favored regions.

Ligands modelling

Crystal structure of clozapine [21] (Fig. 1) was protonated and refined by energy minimization using the Tripos force field [22] of SYBYL 8.0 including the electrostatic term calculated from Gasteiger and Hückel atomic charges [23, 24]. The Powell method available in Maximin2 procedure [18] was used for energy minimization until the gradient value was smaller than 0.001 kcal mol^{-1} $Å^{-1}$. The clozapine nitrenium ion (Fig. 2) was modeled from the crystal structure of clozapine [21], protonated and optimized using the same energy minimization parameters described above.

The resulting molecular mechanics conformations of clozapine and its nitrenium ion were found to be close to the structures optimized by a quantum mechanical calculation (Fig. 3) using the semi-empirical molecular orbital method PM3 [25] of the MOPAC 6.0 program (Quantum Chemistry Program Exchange, Creative Arts Building 181, Indiana University, Bloomington, Indiana 47405, USA).

Molecular docking studies

The binding modes of the ligands for the rat D2 receptor was studied by flexible molecular docking simulations using the GOLD 4.0 program [26]. We defined the putative binding site of the ligands as a 15 Å sphere centered on the CG of Asp 3.32. In fact, this residue is highly conserved in the aminergic G protein-coupled receptors and was proved

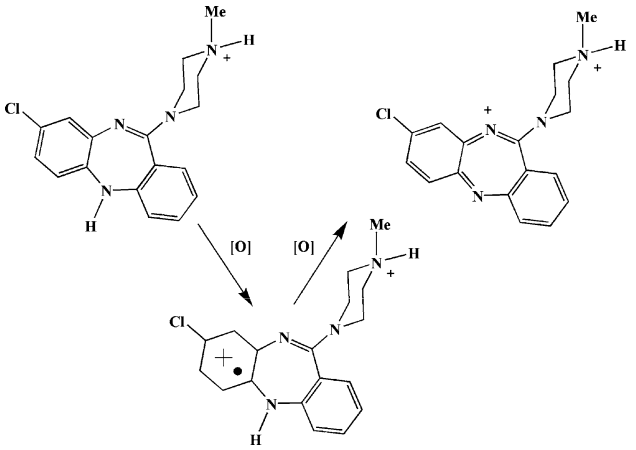

Fig. 2 Formation of nitrenium ion of clozapine after oxidation (after [3]). At pH 7.4, the distal nitrogen is protonated

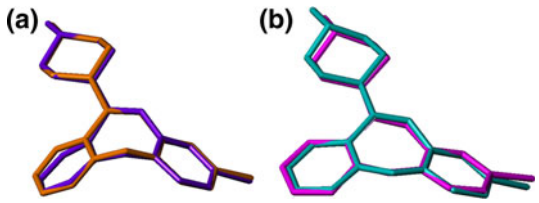

Fig. 3 Superimposition of conformations of **a** clozapine optimized by molecular mechanics (*orange*) and quantum mechanics (*purple*), and **b** clozapine nitrenium ion optimized by molecular mechanics (*magenta*) and quantum mechanics (*cyan*)

to be important in the interactions with the ligands [27]. It is well accepted that the protein and ligand influence the conformation of the other and vice versa. We thus treated the residue side chains of the binding site as flexible. Each flexible side chain was allowed to undergo torsional rotations around one or more of its acyclic bonds from a compilation of the most commonly observed side chain conformations for the naturally occurring residues [16]. Flexibility of the ligands was also considered by using torsion angle distributions extracted from the Cambridge Structural Database (CSD) (http://www.ccdc.cam.ac.uk/products/csd/). These distributions improve the chances of GOLD finding the correct answer by biasing the search towards ligand torsion-angle values that are commonly observed in crystal structures. For each ligand, 20 docking runs were performed. The most stable docking models were selected according to the best-scoring conformation predicted by GoldScore [26]. The docking protocol was validated by redocking carazolol into human $\beta 2$ adrenergic GPCR. The resulting complex was found to be very close to the crystal structure with a RMSD (Root Mean Square Deviation) score of 0.25 Å (Fig. 4). The ligand-D2 receptor complexes derived from docking were further refined in a 200 ps molecular dynamics (MD) simulation using the Tripos force field [22], a temperature of 300 K, and a time

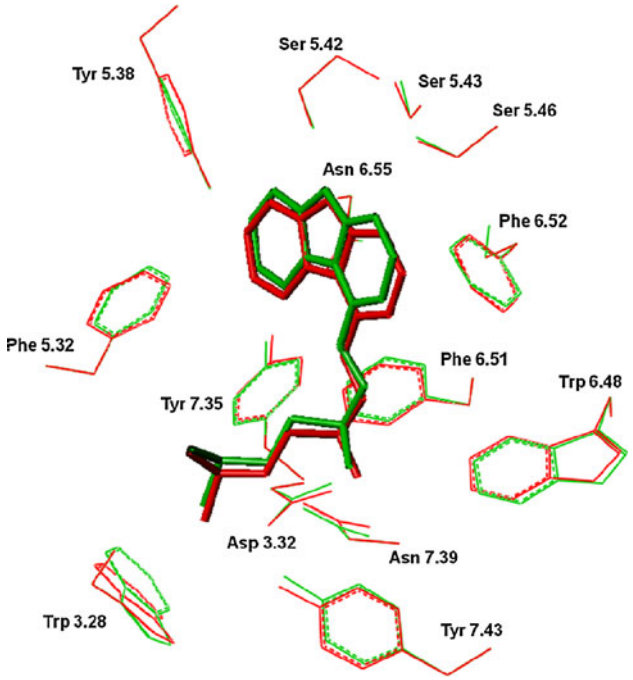

Fig. 4 Superimposition of the β2 adrenergic GPCR-carazolol docking model (*green*) and the crystallographically determined β2 adrenergic GPCR-carazolol complex (*red*)

step of 1 fs. The average structures of the last 190 ps of MD were subsequently energy-minimized using the Powell method available in Maximin2 procedure [18] with the Tripos force field [22], a dielectric constant of 4.0, and a nonbond distance cut-off of 12 Å until the gradient value was smaller than 0.01 kcal mol^{-1} $Å^{-1}$.

Ligands electrostatic potential

The electrostatic potential of clozapine and its nitrenium ion was evaluated by the MOLCAD™ module [28] of SYBYL® 8.0 using the charge distribution calculated from the semi-empirical molecular orbital method PM3 [25].

Results and discussion

The affinity of different tricyclic derivatives tested in normal incubation buffer is found in a similar sequence to that reported in the literature (Table 1; Fig. 5a, c). In the presence of a mixture of HRP/H_2O_2, a strong decrease of binding affinity for D2 receptor is observed for clozapine (Fig. 5b) and olanzapine, two drugs with a diazepine ring while the binding of loxapine (Fig. 5d) and JL13, two oxygen isosteres, and also the thiazepine analogue clothiapine is slightly affected in oxidative conditions. This fact is in agreement with our previous data obtained in different experimental procedures for testing oxidative potential [4, 5] and reports by other teams [6, 29]. The oxidative effect of HRP/H_2O_2 mixture on binding interaction can be prevented by addition of ascorbic acid to the incubation medium.

These results show that the distal nitrogen is less likely affected by the oxidative conditions because loxapine or related analogues presents a similar affinity in both conditions. If the distal nitrogen was transformed in its *N*-oxide the binding would diminish for all of these compounds.

It is known that the oxidation of the tricyclic ring of clozapine generates a nitrenium ion (Fig. 2) [3]. This chemical entity should be responsible for the modification of the binding interactions. The nitrenium ion can be delocalized on the tricyclic ring (Fig. 2) giving different forms in equilibrium [3, 30]. The presence of a *N*-methyl group on the diazepine seems to limit the formation of a nitrenium since the affinity of JL15 in both conditions are quite similar.

In order to understand the difference observed in terms of affinity, the binding mode of clozapine and its nitrenium ion was explored by molecular docking analysis. As shown in Fig. 6a, the docking of clozapine into rat D2 receptor model revealed a binding mode very similar to that observed in the work of Selent et al. [31]. However, it is different from those published by Hjerde et al. [32] and Kalani et al. [33]. These differences are the consequence of structural variations between the human β2 adrenergic receptor and bovine rhodopsin (PDB entry 1F88). Indeed, the binding site of the human β2 adrenergic receptor is narrower because of the displacement of the transmembrane domain TM5 toward the receptor's main axis. The binding mode of clozapine is based on three types of interactions. Firstly, the protonated nitrogen atom of the piperazine ring is shown to form an ionic bond with the carboxyl oxygen of the Asp 3.32 side chain. Secondly, a hydrogen bond between the hydroxyl group of Ser 5.46

Table 1 Binding affinities of clozapine and related analogues on rat D2 receptors in normal and oxidative conditions (Ki in nM; $n \geq 3$)

Compounds	Atom or group of the central ring	Incubation buffer	Incubation buffer plus HRP/H_2O_2 mixture
Clozapine	NH	204 ± 64	>3,000
Loxapine	O	21.4 ± 5.7	33.3 ± 3.0
Clothiapine	S	16.7 ± 1.8	47.9 ± 25.5
Olanzapine	NH	20.6 ± 6.0	>10,000
JL13	O	1,780 ± 368	2,597 ± 113
JL15	N-Me	26,780 ± 6,732	24,340 ± 12,888

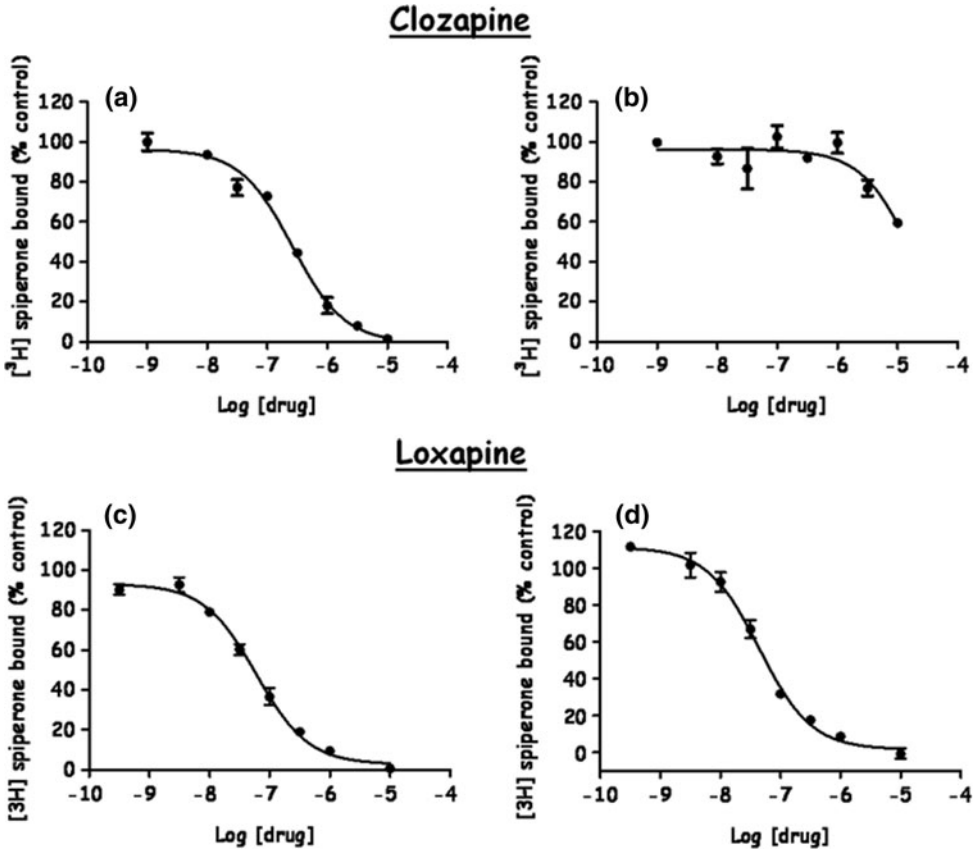

Fig. 5 Representative displacement curves in normal (**a**, **c**) and in oxidative conditions (**b**, **d**) for clozapine and loxapine

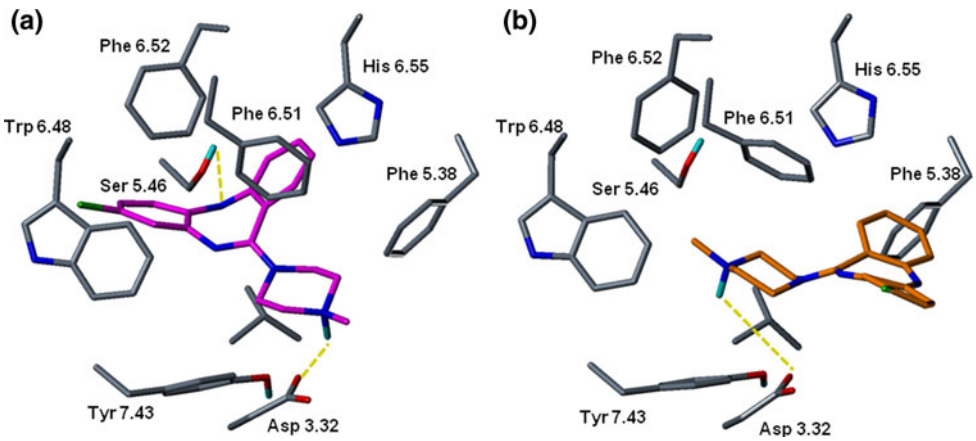

Fig. 6 Binding mode of **a** clozapine and **b** its nitrenium ion in rat D2 receptor. The hydrogen and ionic bonds are indicated by *yellow dashed lines*

and the nitrogen N5 results in a favourable sterically orientation of the tricyclic ring system in the binding site. Finally, this tricyclic system is demonstrated to stabilize the ligand binding by interactions with an aromatic network formed by residues of the transmembrane domains TM5 and TM6 (Phe 5.38, Trp 6.48, Phe 6.51, Phe 6.52 and His 6.55).

The docking of the clozapine nitrenium ion in the same binding site disclosed a ligand position very different from

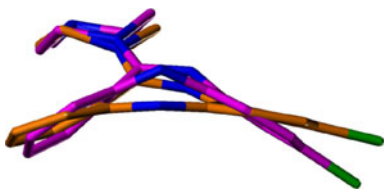

Fig. 7 Superimposition of optimized conformations of clozapine (*magenta, green and blue*) and its nitrenium ion (*orange, green and blue*)

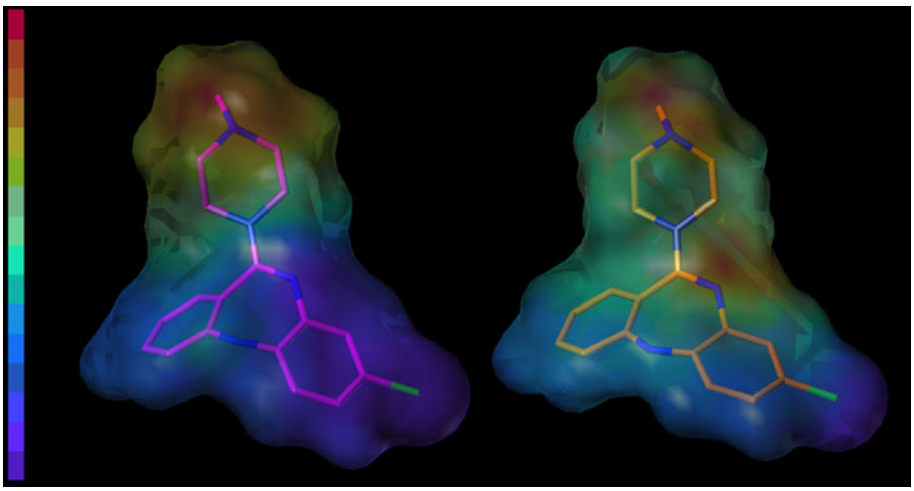

Fig. 8 Electrostatic potential of clozapine (*left*) and its nitrenium ion (*right*). The electrostatic map shows the electron-rich (*purple*) to the electron-deficient (*red*) regions of the compounds

the previously described complex with clozapine (Fig. 6b). The tricyclic ring system is shown to flip in the opposite direction, thereby reducing the interactions with the binding pocket. In fact, the hydrogen bond with the residue Ser 5.46 is not possible and the ionic bond with the residue Asp 3.32 appears to be weaker with a distance of 3.8 Å (versus 2.1 Å for clozapine). Moreover, the tricyclic system is unable to find favourable contacts with the aromatic network.

This decrease in affinity for the binding site can be explained by two major modifications in the tricyclic ring system. Firstly, the nitrenium ion formation leads to a more planar and more extended conformation of the ring system (Fig. 7) which, consequently, is sterically unable to lie in the immediate vicinity of the aromatic network of the binding site. Secondly, the delocalization of the positive charge in the tricyclic system modifies the electrostatic potential of the ring system (Fig. 8). This electronic change seems to be also unfavourable for interactions with the aromatic residues of the binding site. Nevertheless, the lack of binding affinity of the nitrenium ion might be also explained by a desolvation penalty higher than that of clozapine due to the presence of a double positive charge. Indeed, contribution of desolvation to binding affinity was proposed in some protein–ligand complexes [34–37].

Independently the possible role of these oxidized entities in toxicological aspect, it appears that an oxidant character of the extracellular media can greatly influence the activity of drugs like clozapine by formation of such oxidized entities. The presence of H_2O_2 coming from brain mitochondria [38, 39] is demonstrated in the vicinity of neuronal populations [40, 41] and participates to brain cell signalling [39]. Thus, combined with a peroxidase [42] or related enzymes, such oxidative system can greatly modify the pharmacodynamic properties of a drug like clozapine. Otherwise, molecules sensitive to oxidation can be also underevaluated in drug development when such parameter is not adequately estimated. In order to limit these phenomena, ascorbate is added in incubation buffer [43] and thus can minimize the nitrenium formation. Nevertheless, in pathophysiological conditions when the redox potential of the extracellular fluids can vary greatly, the interaction of the drug with the target can be modified consequently. This is not so hypothetical since severe disorders like schizophrenia are frequently associated with neurodegenerative processes and inflammatory events [44–46]. In another context, food deprivation is recently shown to affect brain oxidation status in rat especially with a significant increase in H_2O_2 production [47].

In conclusion, we report experimentally and theoretically the negative impact of nitrenium ion formation for D2 receptor affinity of oxidation-sensitive compounds. This effect appears to be related to a combination of the electronic and geometric changes in the tricyclic moiety that prevent the compound to find the ideal position for a better interaction with the receptor. Finally, in the context of atypicality it could be tempting to involve the nitrenium formation as part of the fast dissociation process.

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