

Changes in A_{2A} adenosine receptor parameters in patients affected by bipolar disorders: Correlation with antipsychotic dosage and severity of illness

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ABSTRACT

Typical antipsychotics, potent D₂ dopamine receptor antagonists, are the most commonly used drugs in the treatment of bipolar disorders. In the central nervous system, the discovery of antagonistic interactions between A_{2A} adenosine receptors and D₂ dopamine receptors suggests that the adenosine system may be involved in the pathogenesis of different psychiatric disorders and in the therapeutic effectiveness of antipsychotic drugs. Previously, we have demonstrated an increase in A_{2A} receptor expression and agonist affinity in platelets from psychotic patients treated with haloperidol. This result suggests that there is also a structural and functional interaction between A_{2A} and D₂ receptors in peripheral cells. In this work, we investigated the effect of different doses of typical drugs on A_{2A} adenosine receptor binding and correlated these parameters with the severity of symptoms. We demonstrated, for the first time, that there was a strong correlation between A_{2A} receptor affinity constant values (K_d) and drug doses in psychotic patients with a moderate severity of illness and moderate psychotic symptoms. The correlation was completely lost in patients with severe illness and severe psychotic symptoms. These results demonstrated that in platelets of patients affected by psychosis, typical antipsychotics modulated A_{2A} receptor binding parameters; this regulation is dependent on the degree of D₂ receptor occupancy in relation to the severity of psychotic symptoms, suggesting A_{2A} receptors are a peripheral marker for individual therapy effectiveness.

Keywords: Bipolar Disorders; Human Platelets; A_{2A} Adenosine Receptors; Typical Antipsychotics; Severity of Illness; A_{2A}/D₂ Functional Interaction

1. INTRODUCTION

Bipolar disorder (BD) is a psychiatric diagnosis that describes a category of mood disorders defined by the presence of one or more episodes of abnormally elevated energy levels, cognition, and mood with or without one or more depressive episodes. The elevated moods are clinically referred to as mania that can sometimes lead to such psychotic symptoms as delusions and hallucinations (DSM-IV-TR, American Psychiatric Association, 2000). Adenosine system has been involved in maniac symptoms and in particular in kindling phenomena that are a valuable model to explain the pathological activation, increase of energy and recurrence of maniac episodes [1]. The role of adenosine in the control of mood in BD stems from different lines of evidences: 1) during kindling phenomena adenosine is released and in several animal models it has been demonstrated that the use of adenosine agonists may have anti-kindling properties [2]; 2) caffeine, that is a non-selective antagonist for both A₁ and A_{2A} adenosine receptor (AR) subtypes, has a mania-like stimulant effects worsening the course of seasonal BD, causing a persistent state of arousal and an exacerbation of maniac symptoms [3-6]; 3) in BD an increase in purinergic turnover has been described [7] and the use of xantine-oxidase inhibitors as anti-maniac agents have been suggested [8,9].

The involvement of adenosine in the control of maniac behaviour first arose based on its tight interaction with dopamine systems [10-13]. The functional and structural interaction between A_{2A} ARs and D₂ dopamine receptors (D₂ DRs) have been yet demonstrated since several years. By the means of different methodological approaches, such as co-immunoprecipitation, FRET/BRET [14,15], biochemical and microdialysis techniques, the existence of A_{2A} AR-D₂ DR heterodimers and higher order receptor heteromers, called receptor mosaics, in the striatopallidal GABA neurons [13,16] has been demonstrated. These heteromers control the excitability of GABAergic neu-

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rons counteracting D₂ DR signalling at multiple effectors [17].

The functional cross-talk between adenosine and dopamine receptors may have important pathophysiological and therapeutic implications in psychiatric diseases associated to dopaminergic dysfunction. The dissection of the molecular mechanisms underlying the regulation of A_{2A} AR expression and functioning represent a crucial aspect in further clarifying the pathophysiological role of adenosine in psychiatric diseases and should be taken into account (1) in developing new drugs as well as (2) in preventing possible side effects of drugs indirectly modulating the adenosine system.

Recently, we have demonstrated both in transfected cell lines [18] and in platelets of patients with BD [19], that typical antipsychotic drugs, acting as D₂ DR antagonists, modulated A_{2A} AR functional responsiveness, suggesting A_{2A} AR agonists as a possible target, in association with D₂ antagonists, in the therapeutic treatment of BD. Based on these results we evaluated the effect of different doses of typical drugs on A_{2A} AR binding parameters in order to identify a possible correlation between the modulation of A_{2A} receptor activity and the degree of D₂ receptor blockade also in relation to the severity of illness and the effectiveness of individual therapy. These results allowed us to identify A_{2A} AR as a peripheral marker of D₂ DR occupancy and of the therapeutic effectiveness of typical drugs.

2. METHODS

2.1. Study Sample

Twenty-four patients affected by BD with psychotic symptoms were recruited from the Department of Psychiatry, University of Pisa, Pisa, Italy. All patients were treated with typical antipsychotic drugs for at least six months. The patients received doses of antipsychotic therapy between 20 to 900 mg/day equivalent of chlorpromazine. The patients met the DSM-IV-TR diagnostic criteria (American Psychiatric Association, 2000) for BD and were between the ages of 18 and 65 years. Subjects who were pregnant or had unstable medical conditions were excluded. All of the patients gave their informed consent to participate in the study. The study was approved by the local Ethical Committee in accordance with the Declaration of Helsinki (1996), and with the Guidelines of the Good Clinical Practice (1995). The control group included 32 healthy volunteers with no history of mental disorders, alcoholism or drug abuse, and with no medical illnesses, as determined by clinical interviews.

2.2. Clinical Assessments

The severity of illness of each patient was evaluated us-

ing the Clinical Global Impression Scale (CGI) [20]. The level of symptoms during the week preceding discharge was assessed using the 18-item version of the brief psychiatric rating scale (BPRS) [21]. Clinical information was collected directly from the patients and from at least one close relative as a co-informant. Medical health was documented by reviewing the medical history and through a physical examination. Three resident psychiatrists with clinical research experience performed the diagnostic and psychopathological assessments. Two senior psychiatrists (LDO and AC) who were not directly involved in the assessments confirmed the diagnoses.

2.3. Materials

Labelled and unlabelled-(2-[7-amino-2-(2-furyl) [1,2,4] triazolo [2,3-a] [1,3,5] triazine-5-yl-amino]ethyl)phenol (ZM₂₄₁₃₈₅) were from Tocris Cookson (UK). All other chemicals were supplied by standard commercial sources.

2.4. Platelet Isolation

Blood samples (30 ml) from healthy volunteers and patients were collected in sodium citrate anticoagulant (1:6 dilution, 2.2% sodium-citrate, and 1.2% citric acid), and platelet-rich plasma (PRP) was obtained by low-speed centrifugation at 200 × g for 20 min at room temperature. The PRP was then centrifuged at 2000 × g for 15 min at room temperature to precipitate platelets. The platelets were then washed once in 40 - 50 ml of an ice-cold physiological saline solution (isotonic, 0.9% NaCl) and pelleted at 15,000 × g for 15 min at 4°C.

2.5. Preparation of Platelet Membrane Suspensions

Platelet membranes were prepared from 32 healthy volunteers and 24 patients as previously described [19,22]. Briefly, platelets were suspended in Tris-buffer A (50 mM Tris-HCl, 20 mM EDTA, 150 mM NaCl; pH 7.4 at 4°C) and centrifuged at 17,500 × g for 15 min at 25°C. The washed platelets were suspended in ice-cold hypotonic buffer B (5 mM Tris-HCl, and 5 mM EDTA, pH 7.4 at 4°C) and homogenized with a Polytron for 30 second before centrifugation at 35,000 × g for 15 min at 4°C. The resulting pellet was re-suspended in membrane buffer (50 mM Tris-HCl, pH 7.4, 0.8 mM EDTA, 0.16 mg/ml benzamidine, 0.2 mg/ml bacitracine, and 0.02 mg/ml trypsin inhibitor), rapidly frozen in liquid nitrogen and stored at -80°C until it was used.

2.6. [³H] ZM₂₄₁₃₈₅ Binding Assay

A_{2A} AR equilibrium binding parameters were determined by saturation binding studies using the selective A_{2A} AR antagonist [³H]ZM₂₄₁₃₈₅ [19]. Saturation binding experi-

ments were performed incubating platelet membranes (50 μ g) in binding buffer (50 mM Tris-HCl, and 0.8 mM EDTA, pH 7.4) in the presence of five different [3 H] ZM₂₄₁₃₈₅ concentrations ranging from 0.2 to 10 nM at 4°C for 1 h. Non-specific binding was determined in the presence of 1 μ M unlabelled ZM₂₄₁₃₈₅.

2.7. Data Analysis and Statistic

Scatchard analysis of radioligand saturation binding data was performed using the non-linear multipurpose curve-fitting computer program Graph-Pad Prism (GraphPad, San Diego, CA).

Since K_d and B_{max} values are not normally distributed, all of the statistical analyses were conducted on their logarithmic transformations. To compare the mean levels of these variables in control vs. patient independent samples, the Student's t test was utilized. A p-value of <0.05 was judged as statistically significant. All data are presented as mean \pm standard deviation.

Linear regression analyses and bivariate Pearson's correlation coefficients were performed in order to evaluate the relationships between the K_d values and the chlorpromazine equivalent of drug in relation to patient characteristics (severity of illness, total PBRs and factor scores).

All of the analyses were carried out using SPSS, version 14.0, by means of personal computers.

3. RESULTS

3.1. Clinical Characteristics of BD Patients

No gender differences were found between the control (male: 81.3%) and BD patient (male: 83.3%) groups.

The mean BPRS total scores \pm SD by the patients was 48.46 ± 15.77 , while the score of each factor was the following: anxiety-depression (ANDP) = 3.27 ± 1.28 , anergia (ANER) = 1.50 ± 0.67 , thought disturbance (THOT) = 2.52 ± 1.73 , activation (ACTV) = 3.54 ± 1.72 , and hostile suspiciousness (HOST) = 2.80 ± 1.67 .

3.2. A_{2A} AR Equilibrium Binding Parameters in Platelets from BD Patients under Treatment with Typical Antipsychotics

The A_{2A} AR equilibrium binding parameters were evaluated in human platelets from healthy volunteers, (N = 32) and patients with BD (N = 24) under chronic treatment with typical antipsychotics. Scatchard analysis of the [3 H] ZM₂₄₁₃₈₅ saturation binding data demonstrated that in control platelets, the radioligand bound a homogenous population of binding sites with an affinity constant value (K_d) and a maximum density of binding sites (B_{max}) of 4.59 ± 1.79 nM (**Figure 1 (a)**) and 244.97 ± 76.93 fmol/mg proteins (**Figure 1 (b)**), respectively. To evalu-

ate the effect of antipsychotic treatment on the A_{2A} AR binding parameters, [3 H]ZM₂₄₁₃₈₅ saturation analysis was performed on platelet membranes obtained from 24 BD patients treated with typical antipsychotic drugs for at least six months. In BD patients, [3 H]ZM₂₄₁₃₈₅ bound a homogeneous population of binding sites with a K_d and B_{max} value of 1.90 ± 1.39 nM (**Figure 1 (a)**) and 270.22 ± 150.94 fmol/mg of proteins (**Figure 1 (b)**), respectively.

As shown in **Table 1**, a significant difference in the K_d values between the control and patient groups was observed. On the contrary, in BD patients treated with typical antipsychotics, no significant differences in the B_{max} value were observed with respect to controls.

To evaluate if the variability in the K_d of the A_{2A} AR parameters observed in the BD patients was related to the dosage of the administered typical antipsychotic, statistical correlation analyses were performed. Considering the whole group of patients, no significant correlations were detected between the K_d values and the equivalent of chlorpromazine, and between the K_d and the clinical

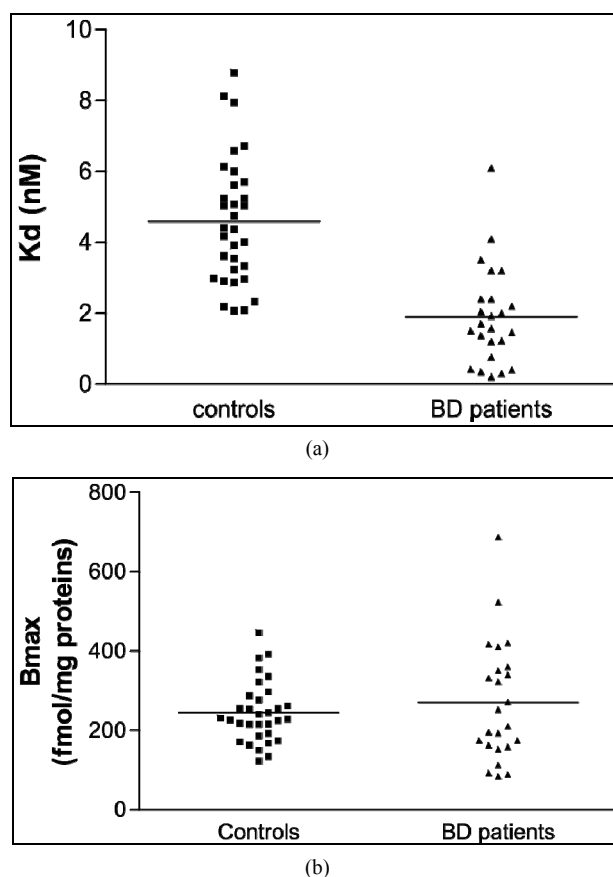


Figure 1. The figures display individual results for the [3 H] ZM₂₄₁₃₈₅ K_d (A) and B_{max} (B) values obtained in platelets from healthy volunteers (controls; N = 32) and BD patients (N = 24). The horizontal lines represent the mean values \pm SEM from healthy volunteers and patients.

Table 1. Comparison between the mean levels of Ln K_d and Ln B_{max} in the controls (a) vs BD patients; (b) Student's t test.

A_{2A} binding parameters	Mean \pm SD	t	Sig. (2-tailed)
Ln K_d	(a) 1.45 ± 0.40	6.321	0.000
	(b) 0.33 ± 0.89		
Ln B_{max}	(a) 5.45 ± 0.31	0.066	0.948
	(b) 5.45 ± 0.58		

patient characteristics (CGI, total BPRS scores and factor scores).

Nevertheless, after subdividing the patients into two groups on the basis of the severity of illness (CGI \leq 4: moderate ill; CGI $>$ 4: severe ill), a significant negative correlation between the A_{2A} AR K_d values and the equivalent of chlorpromazine dose was detected only in the group of patients with moderate illness (see **Table 2**).

Moreover, a negative correlation between the A_{2A} AR K_d values and the equivalent of chlorpromazine dose was detected after subdividing the patients into two groups on the basis of the median of total BPRS scores and the

Table 2. The Bivariate Pearson's correlation between the A_{2A} AR K_d values and the equivalent of chlorpromazine in two groups of BD patients with moderate and severe psychotic disturbance, respectively.

	Moderate illness	Severe illness
CGI	R = -0.755 p = 0.001 n = 14	R = -0.202 p = 0.551 n = 11
Total BPRS	R = -0.794 p = 0.002 n = 12	R = -0.075 p = 0.817 n = 12
THOT	R = -0.669 p = 0.017 n = 12	R = -0.182 p = 0.570 n = 12
OST	R = -0.589 p = 0.057 n = 14	R = -0.219 p = 0.472 n = 13

median of factor III (THOT) and factor V (OST) BPRS scores (see **Table 2**).

In the relative scattergrams (**Figure 2**), we can observe the different slopes of the straight-line for moderate and severe psychotic disturbances.

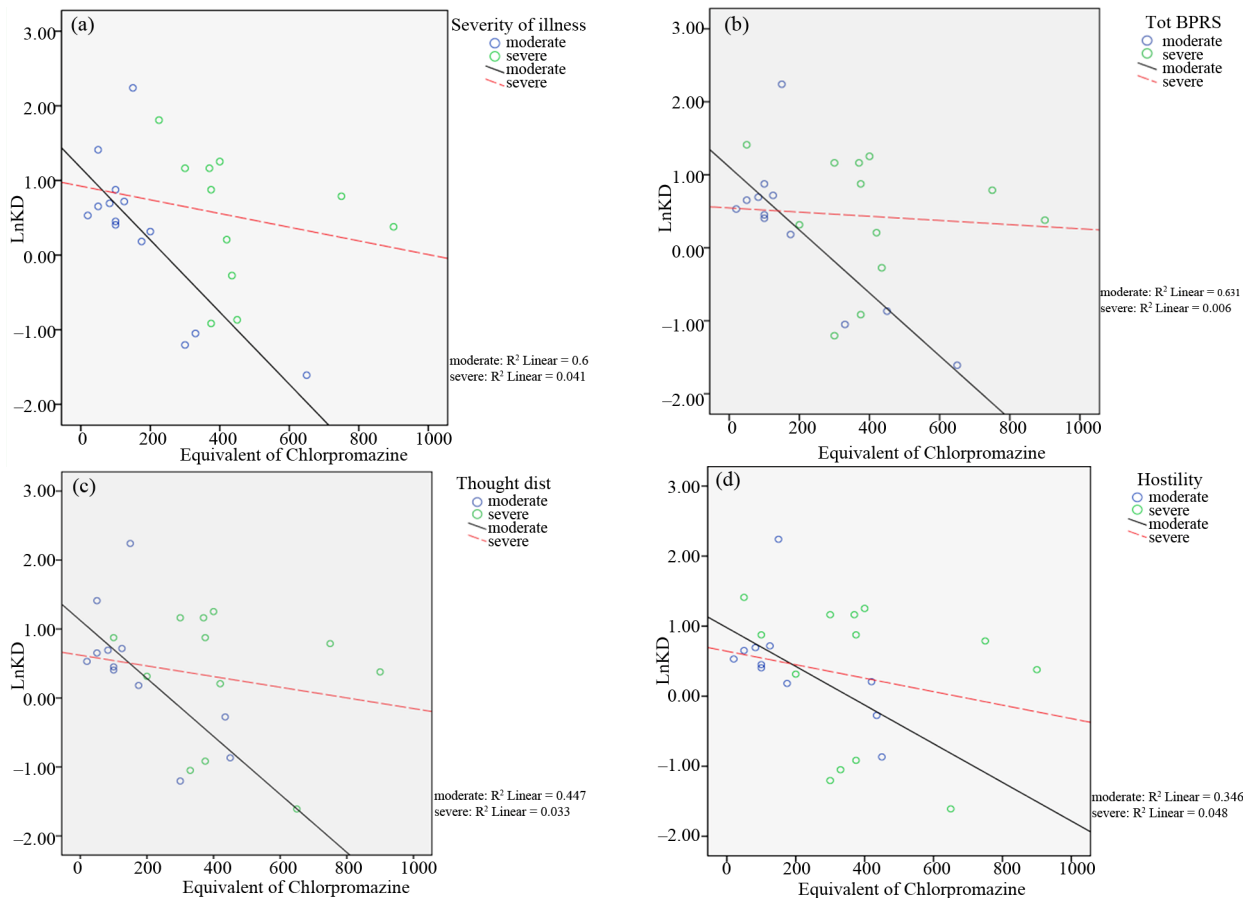


Figure 2. Scattergrams obtained from bivariate Pearson's correlation between A_{2A} AR K_d values and the equivalent of chlorpromazine in the two groups of BD patients with moderate and severe psychotic symptoms. Patient classification was performed on the basis of CGI scores (4 = moderate; $>$ 4 = severe; panel a), the median of total BPRS scores (median = 43.50; panel b), the median of THOT factor scores (median = 1.63; panel c) and the median of HOS factor scores (median = 2.33; panel d).

4. DISCUSSION

In this work, we demonstrated that typical antipsychotic drugs, acting as D₂ DR antagonists, selectively affected the A_{2A} AR affinity constant value in human platelets of BD patients under chronic treatment with these drugs. The main finding of these data is that administered typical drugs induced a reduction in A_{2A} AR K_d values in a dose-dependent-manner, demonstrating the existence of a correlation between D₂ DR occupancy and A_{2A} AR regulation induced by the dopamine system.

Although blocking of the D₂ DR remains central to the therapeutic properties of antipsychotics, these drugs appear to act by mechanisms involving other neurotransmitter systems, including serotonin and adenosine [23, 24]. The involvement of the adenosine system and of the A_{2A} ARs in psychiatric diseases [12,25-28], including BD [3-6] has been demonstrated and it has been related to a functional cross-talk with the dopaminergic system, in particular with the D₂ DRs. D₂ DRs are highly expressed in striatopallidal neurons, where they co-localize with adenosine A_{2A} ARs [29], presumably forming functional heterodimers in which adenosine and dopamine receptors mutually regulate their responses [14,15,30-33].

In a previous work [19], we demonstrated that typical antipsychotics, but not atypical ones, induced an up-regulation of A_{2A} AR expression and functional response in the platelets of BD patients. We demonstrated in particular that: 1) A_{2A} and D₂ receptors are co-expressed in platelets and interact to form functional heterodimers in which the response of one receptor may be modulated by the selective block of the other, and 2) typical and atypical antipsychotics, which show a different mechanism of action at the molecular level, may differently control the functional regulation of A_{2A} AR. A crucial point that distinguishes different classes of antipsychotics is the degree of dopamine D₂ DR occupancy and the kinetics of drug receptor-dissociation, on which the drug response and some unpleasant side effects depend. Typical antipsychotics, such as haloperidol, have a high D₂ DR occupancy and slow dissociation rates that are likely connected to the extrapyramidal side effects [34,35] of these drugs.

On the contrary, clozapine, an atypical drug, shows a much lower occupancy and a fast dissociation rate; this may explain its freedom from extrapyramidal side effects [36,37]. These differences in the activity profiles of the two antipsychotic classes may be at the root of the specific effects observed in the A_{2A} regulatory mechanisms.

In order to investigate whether A_{2A} AR could be used as a marker of D₂ receptor occupancy in relation to therapy efficacy, we aimed to evaluate the regulation of A_{2A} AR that is induced by different doses of typical drugs. The study was performed by assessing A_{2A} AR equilibrium-binding parameters in the platelets of patients af-

ected by bipolar disorders, all under the treatment of typical drugs for at least six months.

The results confirmed that in BD patients, a reduction in A_{2A} AR K_d values had occurred, demonstrating that the typical antipsychotics affected A_{2A} AR ligand affinity by increasing its functional activity. On the contrary, with respect to the previous work, increasing the number of patient groups resulted in no significant differences in the maximal density of A_{2A} AR binding sites. These results suggest that the block of the D₂ receptor mainly affects the A_{2A} AR conformational state rather than its total number. These data have been also confirmed in a model of CHO cells co-transfected with both A_{2A} and D₂ receptors [18]. Statistical analyses were also performed in order to evaluate the existence of a correlation between the changes in the A_{2A} K_d values and the drug doses, expressed as an equivalent of chlorpromazine. Interestingly we demonstrated that in the BD patient groups that there was no statistically significant correlation between the A_{2A} AR K_d values and the equivalent of chlorpromazine. Additionally, when the patients were grouped on the basis of severity of illness as “moderate illness” (CGI value ≤ 4) and “severe illness” (CGI > 4), a significant negative correlation between the two parameters was detected only in the moderate illness group of the patients. On the other hand, this correlation was lost in the group of patients with a high severity of illness. Moreover, the same results were obtained when the patients were grouped on the basis of the median of total BPRS scores, and the median of two BPRS factor scores, and in particular the factor III (Thought disturbance) and the factor V (Hostile suspiciousness). These BPRS factors are the main representative of psychotic symptoms. In detail, factor III includes four items: conceptual disorganization, grandiosity, hallucinatory behaviour and unusual thought content. Factor V includes three items: hostility, suspiciousness and uncooperativeness, all symptoms of psychotic behaviour. Our results can then suggest that the regulation of A_{2A} AR in response to antipsychotics may be a new marker for monitoring therapy efficacy, and particularly in relation to psychotic symptoms. In addition, the results suggest that the degree of the D₂ receptor block may have an important role in the A_{2A} AR regulatory mechanisms only in responder patients with respect to non-responder patients. The lack of A_{2A}-D₂ cross-regulation in non-responder patients could be explained by the idea that in non-responder patients an alteration of the dopaminergic system response had occurred. This hypothesis may be supported by the primary persistence of negative symptoms in these patients. Furthermore, it is also likely that the impairment of the responsiveness of the dopamine receptor caused, in turn, an alteration in the cross-regulation between adenosine and dopamine receptors. To dissect this issue a direct monitoring of D₂ receptor occupancy and of D₂ receptor

functional responsiveness in non-responder vs responder patients should be evaluated. Unfortunately, the evaluation of receptor D₂ occupancy is not detectable by radioligand binding in peripheral human blood cells that express the receptors at low levels. In fact, all of the studies aimed to assess the degree of D₂ receptor occupancy following antipsychotic administration have been performed in animal models [38] or in the human brain using PET/SPECT technology [39-42]. In this context, since D₂ receptor occupancy has been identified as an important aspect for individual therapeutic responsiveness and for the appearance of side effects, the A_{2A} AR binding parameters, which are quantifiable in human platelets, may represent an indirect index to evaluate the degree of D₂ receptor occupancy for individual patients. This may be a useful means to seek therapy for maximum effectiveness and fewer side effects.

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