JPET Fast Forward. Published on October 31, 2006 as DOI: 10.1124/jpet.106.111203

JPET#111203

ZM241385, DPCPX and MRS1706 are inverse agonists with different relative intrinsic efficacies on constitutively active mutants of the human adenosine A_{2B} receptor

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JPET#111203

Running title: Inverse agonist activity on CAM adenosine A_{2B} receptors

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Number of text pages: 32

Number of tables: 4

Number of figures: 4

Number of references: 36

Number of words in abstract: 248

Number of words in introduction: 652

Number of words in discussion: 1625

Abbreviations:
GPCR, G protein-coupled receptor; NECA, 5'-*N*-ethylcarboxamidoadenosine; ZM241385, 4-(2-[7-amino-2-(2-furyl[1,2,4]-triazolo[2,3-a[1,3,5]triazin-5-yl-aminoethyl) phenol) ; DPCPX, 8-Cyclopentyl-1,3-dipropylxanthine ; MRS1706, *N*-(4-acetylphenyl)-2-[4-(2,3,6,-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl) phenoxy] acetamide ; 3-AT, 3-aminotriazole; wt, wild-type; CAM, constitutively active mutant.

Recommended section:

Cellular and Molecular

Abstract

The human adenosine A_{2B} receptor belongs to class A G protein-coupled receptors (GPCRs). In our previous work, constitutively active mutant (CAM) human adenosine A_{2B} receptors were identified from a random mutation bank. In the current study, three known A_{2B} receptor antagonists, ZM241385, DPCPX and MRS1706 were tested on wild-type and 9 CAM A_{2B} receptors with different levels of constitutive activity in a yeast growth assay. All three compounds turned out to be inverse agonists for the adenosine A_{2B} receptor as they were able to fully reverse the basal activity of 4 low level constitutively active A_{2B} receptor mutants and to partially reverse the basal activity of 3 medium level constitutively active A_{2B} receptor mutants. We also discovered 2 highly constitutively active mutants whose basal activity could not be reversed by any of the three compounds. A two-state receptor model was employed to explain the experimental observations; fitting these yielded the following relative intrinsic efficacies for the three inverse agonists, ZM241385, DPCPX and MRS1706: 0.14 ± 0.03, 0.35 ± 0.03 and 0.31 ± 0.02, respectively. Moreover, varying L, the ratio of active versus inactive receptors in this model, from 0.11 for mutant F84L to 999 for two highly constitutively active mutants yielded simulated dose-response curves that mimicked the experimental ones. This study is the first description of inverse agonists for the human adenosine A_{2B} receptor. Moreover, the use of receptor mutants with varying levels of constitutive activity enabled us to determine the relative intrinsic efficacy of these inverse agonists.

Introduction

G-protein-coupled receptors (GPCRs) constitute a large superfamily of transmembrane proteins which represent the target for nearly half of the marketed drugs (Drews, 2000; Hopkins and Groom, 2002). One of the GPCR family members, the adenosine receptor, is a group of widely distributed receptors which is composed of four receptor subtypes: A_1, A_{2A}, A_{2B} and A_3. Of these, the adenosine A_{2B} receptor has been implied in many physiological functions (Ralevic and Burnstock, 1998; Feoktistov et al., 1998; Holgate, 2005; Yaar et al., 2005) and adenosine A_{2B} receptor antagonists may be used in the treatment of asthma, type-II diabetes, Alzheimer's disease and cystic fibrosis (Feoktistov et al. 1998; Volpini et al. 2003).

The adenosine A_{2B} receptor is also referred to as the low affinity receptor due to its very modest affinity for the endogenous ligand adenosine. In a VA13 human fibroblast cell line expressing adenosine A_{2B} receptors, adenosine caused an increase in the production of cyclic AMP with an EC_{50} value of 15 μM (Bruns, 1980). Despite elaborate synthesis efforts, a long-established reference compound, NECA still has the highest affinity, 360 nM, for the human adenosine A_{2B} receptor expressed in HEK-293 cells, but it lacks selectivity (De Zwart et al., 1998; Fredholm et al., 2001). Recently a new series of non-ribose agonists for adenosine receptors was described (Chang et al., 2005) including several highly potent agonists for the adenosine A_{2B} receptor (Beukers et al., 2004a). LUF5835 e.g. was a full agonist with an EC_{50} value of 10 nM. Still none of these agonists was truly selective for the A_{2B} receptor.

In contrast, several selective antagonists have been identified for the adenosine A_{2B} receptor. For example, the xanthine amide derivatives MRS1668, MRS1706 and

MRS1754 are relatively new selective antagonists for the adenosine A_{2B} receptor with K_i values in the nanomolar range (Kim et al., 2000). Except for these new antagonists, prototypic adenosine receptor antagonists such as ZM241385, DPCPX, XAC and CGS15943 have a good to reasonable affinity for the adenosine A_{2B} receptor but are not selective versus other subtypes of adenosine receptors (Klotz et al., 1998; Ongini et al., 1999; Alexander et al., 1996).

The phenomenon of constitutive receptor activity i.e. receptor signaling in the absence of agonists allows the discrimination between neutral antagonists and inverse agonists. Although the therapeutic implications remain as yet unclear, many clinically used drugs turn out to be inverse agonists rather than neutral antagonists (Costa and Cotecchia, 2005; Bond and IJzerman, 2006). Whether the currently known antagonists of the adenosine A_{2B} receptor possess inverse agonistic properties is unknown as constitutive activity for the wild-type (wt) adenosine A_{2B} receptor has not been reported yet.

In our previous work, we identified constitutively active mutant (CAM) adenosine A_{2B} receptors from a random mutation bank using a robust yeast selection assay (Beukers et al. 2004b; Beukers and IJzerman, 2005). These yeast cells were genetically engineered to not only communicate with human GPCRs, but also identify (constitutively) active receptors (Pausch, 1997; Dowell and Brown, 2002). To this end the signal transduction pathway of yeast was coupled to the production of the essential amino acid, histidine. Yeast cells expressing gain-of-function or constitutively active adenosine A_{2B} receptors were identified through their ability to grow in histidine-deficient medium.

Since CAM receptors provide a useful tool to discriminate inverse agonists from

neutral antagonists, CAM adenosine A_{2B} receptors identified in our previous study were used in our current study to characterize three A_{2B} receptor antagonists: ZM241385, DPCPX and MRS1706. All three compounds proved to be inverse agonists for the adenosine A_{2B} receptor. The present study is the first to describe inverse agonists for the human adenosine A_{2B} receptor. Moreover, the use of CAM receptors with varying levels of constitutive activity enabled us to determine differences in relative intrinsic efficacy values of these compounds. Finally, mathematical simulation of our experimental data with a two-state receptor model confirmed the intricate connection between the ligands' relative intrinsic efficacy and the receptor's constitutive activity.

Methods

Materials

NECA (5'-*N*-ethylcarboxamidoadenosine) was purchased from Sigma Aldrich, Steinhem, Germany. ZM241385 (4-(2-[7-amino-2-(2-furyl[1,2,4]-triazolo[2,3-a[1,3,5]triazin-5-yl-aminoethyl)phenol)) was a gift from Dr. S.M. Poucher, AstraZeneca Pharmaceuticals, Macclesfield, UK. DPCPX (8-Cyclopentyl-1, 3-dipropylxanthine) was purchased from RBI (Natick, MA, USA). MRS1706 (*N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy] acetamide) was obtained from Tocris Cookson Inc, Bristol, UK. 3-AT (3-amino-[1,2,4]-triazole) was purchased from Sigma Aldrich, St Louis, USA.

A genetically modified yeast *Saccharomyces cerevisiae* strain with the following genotype: *MAT***a***his3 leu2 trp1 ura3 can1 gpa1*Δ::Gα_{i3} *far1*Δ::ura3 sst2Δ::ura3 Fus1::FUS1-HIS3 LEU2::FUS1-lacZ ste2Δ::G418^R was a gift from Dr. S. J. Dowell, GSK (Stevenage, UK). Constitutively active adenosine A_{2B} receptor mutants (CAM) were obtained through random mutagenesis as previously described (Beukers et al., 2004b). These mutants were identified with a screening assay based on yeast growth.

Methods

Yeast growth assay

Nine constitutively active adenosine A_{2B} receptor mutants were selected from a random mutation bank based on their ability to grow on solid or in liquid medium in the

absence of agonists. This growth was independent of endogenous adenosine as the addition of adenosine deaminase did not affect yeast growth (Beukers et al. 2004). Single colonies of these nine CAM receptors were used to guarantee a consistent phenotype.

To perform growth assays on solid agar medium, yeast cells from an overnight culture were diluted to around 400,000 cells/ml (OD_{600} = 0.02) and droplets of 1.5 µl were spotted on growth assay plates containing minimal agar medium, 20 mg/l adenine, 20 mg/l tryptophan, with or without either ZM241385 (1 nM to 0.1 mM), DPCPX (1nM to 0.1 mM) or MRS1706 (0.1 nM to 10 µM). Receptor-independent growth was suppressed through the addition of 7 mM 3-AT. After incubation at 30 °C for 48 hours, the plates were scanned and receptor-mediated yeast growth was quantified with Quantity One imaging software from Bio-Rad (Hercules, CA). The growth rate of yeast was calculated as the density of each spot with a correction for local background on the plate.

For growth assays in liquid medium, an overnight yeast culture was adjusted to OD_{600} = 0.2 and 50 µl was seeded into each well of a 96-wells plate. Every well contained 200 µl minimal medium, 20 mg/l adenine, 20 mg/l tryptophan, 7 mM 3AT with or without the indicated concentrations of ZM241385, DPCPX or MRS1706. The cells were kept at 30 °C for 35 hours and yeast growth in 96-wells plates was automatically recorded using a Genios plate reader (Tecan Inc.). OD_{600} of every well was used to determine yeast growth. Variations in the background OD values were insignificant hence no background correction was carried out in the liquid medium growth assay experiments.

Each experiment was repeated 3 to 5 times. The IC_{50} values and E_{max} values were calculated with Prism version 4.0 (GraphPad Software Inc., San Diego, CA). Relative efficacies of the compounds were calculated as the ratio of maximum response, in this

case the maximun inhibition induced by an inverse agonist divided by that of the full inverse agonist. In this study, ZM241385 exhibited the strongest inverse agonism, thus it was used as full inverse agonist to calculate the relative efficacy of the other two compounds DPCPX and MRS1706.

Schild analysis

NECA concentration-growth curves of the wild-type adenosine A_{2B} receptor were obtained in the absence and presence of ZM241385, DPCPX or MRS1706. Dose ratios (DR) were calculated from the molar NECA concentrations producing a half-maximal response (EC_{50}) in the presence of one of these three compounds divided by the EC_{50} obtained in the absence of these compounds. The DRs were subjected to Schild analysis to determine whether the compounds acted as competitive antagonists (Cheng, 2004):

$$\text{Log}(DR-1) = n\text{Log}[B] - \log K_B$$

In this equation, [B] refers to the molar concentration of ZM241385, DPCPX or MRS1706 and K_B is the equilibrium dissociation constant of the complex of the receptor with one of the compounds. A plot of log (DR-1) values (y-axis) versus logarithm molar concentrations of these compounds (x-axis) yielded a straight line of which the intercept reflects the pK_B or pA_2 value of the compound and the slope (n) reveals whether the compound is a competitive antagonist (n=1) or not ($n \neq 1$).

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Parameter fitting

The two-state receptor model was originally applied to describe the function of ion-channels and adapted to explain the activation of receptors (Leff, 1995). In this study, it is used to interpret and simulate our experimental data. The two-state receptor model is described by three parameters: L, the isomerization constant which is the ratio of the receptor in the active R_a state versus the inactive R_i state; α, the so-called intrinsic efficacy which refers to the affinity of a ligand for the active state of the receptor (R_a) over the inactive state of the receptor (R_i); K_A, the equilibrium dissociation constant of a ligand-receptor complex. This two-state model does not imply any downstream signaling effects. Hence the intrinsic efficacy represented by the α value may still be system-dependent. To avoid confusion we will therefore employ the term relative intrinsic efficacy rather than intrinsic efficacy that is system-independent by definition. To account for downstream signaling effects the equation for the two-state model should be extended with an intervening forcing function as shown in the supplemental data. In such an equation the α values represent the 'true' intrinsic efficacies that are system-independent. We assume that in our yeast system the forcing functions will be identical among the tested mutants. We therefore ignored these forcing functions and used the simple two-state model.

According to the two-state receptor model, the proportion of receptors in the active state can be calculated as:

$$\rho = \frac{\alpha L[A]/K_A + L}{[A]/K_A(1+\alpha L) + L + 1} \qquad \text{equation 1 (Kenakin, 2003)}$$

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In the absence of ligand ([A] = 0) the proportion of receptors in the active state, the constitutively active receptors, can be stated as:

$$\rho_0 = L/(L+1) \qquad \text{equation 2}$$

From these equations the L value, the ratio of activated receptors versus inactivated receptors, can be calculated:

$$L = \rho_0/(1-\rho_0) \qquad \text{equation 3}$$

The value ρ_0 (the proportion of receptors in the active state in the absence of ligand) of each CAM receptor was determined by quantification of spontaneous yeast growth in histidine-deficient medium in the presence of 7 mM 3AT. Yeast growth is positively correlated to the proportion of receptors in the active state, thus yeast growth can be used to calculate the ρ values of the receptors. To determine the ρ values of the different CAM receptors we first defined the two extreme values detected in this study. The lower limit of ρ_0 was set at 0.001 (reflecting 0.1% of receptors in the active state) for the growth obtained upon expression of the wt receptor. The upper limit of ρ_0 was set at 0.999 (which means 99.9% of receptors are active) for the growth obtained upon expression of either one of the two mutant receptors, A18T/A23V/C83Y/A106V/R112S or Q214L/I230N/V240M/V250M/N254Y/T257S/K269stop. These mutants exhibited the highest level of constitutive activity among all the mutants and could not be further activated by addition of agonists such as NECA (data not shown), which suggested that they have reached maximal levels of receptor activation. Thus, the L values of the wt and these two mutants were fixed at 0.001 and 999, respectively (see equation 3).

The ρ values of mutants in the presence and absence of concentrations of ZM241385, DPCPX or MRS1706 were scaled according to two defined extreme values: 0.001 and

0.999. Then the software package Prism was used to perform curve fitting according to equation 1 and to determine the values for L, α and K_A.

Since the two highly CAM receptors A18T/A23V/C83Y/A106V/R112S and Q214L/I230N/V240M/V250M/N254Y/T257S/K269stop could not be inhibited by any of the compounds tested, the growth of these two mutants in the presence and absence of inverse agonist could not be used for curve fitting in Prism.

Simulation of growth curves using MatLab

The pharmacological two-state receptor model was implemented in the software package MatLab version 7.0 (The Mathwork, Inc, Natick, USA) and a graphic interface was composed to facilitate parameter input and to simulate curves. Concentration-proportion (ρ) curves were simulated and visualized with a fixed α and K_A value and variable L values to mimic the experimental curves.

Results

Identification of constitutively active mutants

To select constitutively active mutant A_{2B} receptors, we employed a yeast assay based on growth which was previously described (Beukers et al., 2004b). In brief, human adenosine A_{2B} receptors couple to the endogenous signaling pathway of yeast (Brown et al., 2000) and activate the synthesis of histidine, which allows the yeast cells to grow in histidine-deficient medium. Hence, the growth of these yeast cells and the presence of active adenosine A_{2B} receptors are positively correlated and increased receptor activity results in increased yeast growth.

From our random mutation bank nine previously published A_{2B} receptor mutants with different levels of constitutive activity were selected (Beukers et al., 2004b) and used as screening tools to discover inverse agonists. Among them, the highest levels of constitutive activity were obtained with two multiple mutant receptors, A18T/A23V/C83Y/A106V/R112S and the truncated mutant Q214L/I230N/V240M/V250M/N254Y/T257S/K269stop, missing the C-terminal and transmembrane helix 7. Mutants N36S/T42A, N36S/T42A/T66A and T42A/V54A showed intermediate levels of agonist-independent growth. Finally, three point mutants T42A, F84L, F84S and the double mutant F84L/S95G exhibited relatively low levels of constitutive activity (Table 1).

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Antagonism of ZM241385, DPCPX, and MRS1706 at the wild-type adenosine A_{2B} receptor

The agonistic effect of NECA on the wild-type adenosine A_{2B} receptor was antagonized by ZM241385, DPCPX, and MRS1706 in a concentration-dependent manner (Fig. 1), which resulted in a rightward shift of the concentration-response curve without a change of E_{max}. Schild analysis of this antagonism (n=3) yielded pA_2 values of 7.32 ± 0.29 for ZM 241385, 6.41 ± 0.16 for DPCPX and 7.38 ± 0.18 for MRS1706. The slope of the Schild plot was 0.99 ± 0.03 for ZM241385, 1.04 ± 0.14 for DPCPX and 1.26 ± 0.07 for MRS1706 indicating the competitive interaction of these compounds with the receptor. Table 2 displays a comparison of the pA_2 values of ZM241385, DPCPX and MRS1706 obtained in this study with literature values.

Inverse agonistic properties of ZM241385, DPCPX and MRS1706

Three types of response of the constitutively active mutants were observed when yeast cells expressing CAM A_{2B} receptors were cultured in the presence of ZM241385, DPCPX or MRS1706.

Both the mutants with low and intermediate levels of constitutive activity were inactivated by ZM241385, DPCPX, and MRS1706. Hence these compounds acted as inverse agonists on the constitutively active human adenosine A_{2B} mutants, albeit with varying intrinsic activities and potencies as described in detail below (Fig 2, Table 3).

The basal growth of yeast cells expressing T42A, F84L, F84S or F84L/S95G mutant receptors was dose-dependently reduced by ZM241385, DPCPX and MRS1706 from 91% to 99%. In other words, the basal growth of yeast cells expressing these 4 low CAM

receptors was almost completely inhibited in the presence of 10 μM of any of these inverse agonists. The mutants with intermediate levels of constitutive activity, N36S/T42A, N36S/T42A/T66A and T42A/V54A were inhibited by all three inverse agonists albeit with lower intrinsic activities, ranging from 28% to 79%. None of the tested inverse agonists could, however, completely inhibit the growth of yeast cells expressing any of these 3 medium level CAM receptors.

The multiple mutant A18T/A23V/C83Y/A106V/R112S and the truncated mutant Q214L/I230N/V240M/V250M/N254Y/T257S/K269stop did not respond to ZM241385, DPCPX or MRS1706 at any of the concentrations tested. A representative experiment with ZM241385 is shown in figure 3. Apparently, these two CAM A_{2B} receptors are endowed with robust activity and seem to be locked in an active state.

Among the three compounds, ZM241385 exhibited the strongest intrinsic activity, thus the relative efficacies of the compounds tested were calculated by taking ZM241385 as the reference full inverse agonist. As shown in table 3, the relative efficacy of all three compounds on low level CAM receptors was close to 100%, while the relative efficacy on medium level CAM receptors varied from 44% to 100%.

Point mutants F84L and F84S behaved similar to each other with respect to both the potency and relative intrinsic efficacy of the inverse agonists, indicating that a mutation from phenylalanine to leucine or serine has the same effect. These two single mutants have very low constitutive activity and were completely inhibited by all three inverse agonists. An additional S95G mutation seemed to have no effect on either the constitutive activity or the inverse agonistic effect.

Like the point mutants F84L and F84S, the T42A mutant exhibited relatively low constitutive activity that was completely inhibited by all three inverse agonists. Comparison of the T42A point mutant with the T42A/V54A and the N36S/T42A double mutants showed that the IC_{50} values of the three inverse agonists were at most 3-fold altered when an additional V54A or N36S mutation was present. However, whereas the constitutive activity of the T42A mutant could be completely inhibited by all three inverse agonists, an additional V54A or N36S mutation resulted in a higher level of constitutive activity and could be only partially inhibited. Whereas the N36S mutation did not cause significant changes with respect to the potency of the three compounds, an additional T66A mutation along with the T42A/N36S double mutation increased the IC_{50} values of ZM241385 and DPCPX from 187 nM to 522 nM and from 1.44 μM to 6.25 μM, respectively. Due to the small window, the IC_{50} value of MRS1706 for the T42A/N36S/T66A triple mutant could not be determined. In addition, the level of constitutive activity of this N36S/T42A/T66A triple mutant is increased compared to the N36S/T42A double mutant. The most interesting observation concerning the CAM receptors containing a T42A mutation was that the additional mutations increased not only the constitutive activity of the receptor, but also made the receptor less sensitive towards the inverse agonists.

For ZM241385 and MRS1706, not only the intrinsic activity profoundly decreased on the mutants with increasing levels of constitutive activity, but their potency also slightly decreased. For instance, the IC_{50} values of ZM241385 for the medium level CAM N36S/T42A and T42A/V54A were approximately 2-fold higher than the IC_{50} values for this compound on the low level CAM receptors. In addition, the IC_{50} value of ZM241385

increased another 2.5- to 3-fold for N36S/T42A/T66A which had slightly higher constitutive activity than the other two medium level CAM receptors. Similarly, the IC_{50} values of MRS1706 for medium level CAM receptors were also slightly higher than for low level CAM receptors, varying from 1.5-fold to 4-fold. However, the potency of DPCPX for mutants with increased level of constitutive activity did not increase except for N36S/T42A/T66A triple mutant.

The rank order of potencies of the inverse agonists showed that with the exception of the T42A/V54A double mutant, the IC_{50} values of DPCPX were 8- to 22-fold higher than the values for ZM241385. The IC_{50} values of MRS1706 in turn were 11- to 36-fold lower than the values for DPCPX which is in accordance with the weaker antagonism of DPCPX at the wt adenosine A_{2B} receptor (see Tables 2 and 3).

Estimation of parameters of the two-state receptor model

More than ten years ago the two-state receptor model was introduced to successfully explain some observations of GPCRs in cell lines and in recombinant receptor-expression systems (Leff, 1995); for example a partial agonist in one experimental system could behave as a full agonist in another. In this study, we applied this model to characterize both the constitutive activity of CAM adenosine A_{2B} receptors and the relative intrinsic efficacies of the tested compounds.

Three important parameters: L (the ratio of active receptors R_a versus inactive receptors R_i), α (relative intrinsic efficacy, which reflects the ratio of ligand affinity for R_a over R_i) and K_A (equilibrium dissociation constant of the ligand-receptor complex

(AR_i)) were obtained from the scaled data of 3 to 5 independent experiments. Curve fitting was performed first on dose-responsive curves for each compound on each mutant. Similar α and K_A values were found for each individual compound on different CAM receptors. Therefore we assumed next that a compound has the same α and K_A values for all CAM A_{2B} receptors. Based on this assumption, dose-response curves from all experiments were fitted with the constraint that the α value and K_A value for each compound were shared among the different mutants while the L values of each mutant were allowed to vary. In this way we could partially reduce the otherwise significant standard deviations, observed on low level CAM receptors in particular.

When $\alpha>1$, the ligand will enrich the R_a state and is classified as an agonist; conversely, if $\alpha<1$, the ligand will enrich the R_i state and is classified as an inverse agonist. The α values obtained from the curve fitting procedure as described above were 0.14 ± 0.03, 0.35 ± 0.03 and 0.31 ± 0.02 for ZM241385, DPCPX and MRS1706, respectively. For an inverse agonist the α value is always between 0 and 1, since α is defined as the ratio of receptors in the two different states R_a and R_i, Within this range, a smaller value for α ('more R_i') corresponds to a stronger inverse agonist. Thus, among these 3 inverse agonists, ZM241385 had the lowest relative intrinsic efficacy value rendering it the strongest inverse agonist tested, whereas MRS1706 and DPCPX had comparable relative intrinsic efficacies. This rank order of fitted α values was consistent with the maximal inhibition rate (I_{max}) of the inverse agonists on mutants with medium levels of constitutive activity (Table 3). We also included the relative efficacies of the three compounds in Table 3 setting the value for ZM241385 to 100%.

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Next to the α values also the K_A values were fitted; they were 89 ± 17 nM, 449 ± 146 nM and 30 ± 8 nM for ZM241385, DPCPX and MRS1706, respectively. According to the two-state receptor model, the dissociation constant K_A is proportional to the EC_{50} value ($EC_{50}=K_A(1+L)/[(1/\alpha)+L]$) (Kenakin, 2003). The general rank order of fitted K_A values was DPCPX > ZM241385 > MRS1706, which was in agreement with the IC_{50} data (see Table 3). In addition, when these K_A values were transformed to pK_A values (7.09 for ZM241385, 6.35 for DPCPX and 7.52 for MRS1706), we learned that they were similar to the pA_2 values (7.32, 6.41 and 7.38 for these three compounds) on the wild-type A_{2B} receptor. Thus we felt confident to assume similar K_A values for both wild type and mutant receptors.

The L values, representing the ratio of active versus inactive receptors, of low level and intermediate level CAM adenosine A_{2B} receptors were determined as described in the Materials and Methods section. The fitted L values differentiated into 2 groups (Table 4): Low level CAM receptors have L values around or slightly smaller than 0.2; intermediate level CAM receptors have L values ranging from 1.38 for the T42A/V54A mutant to 1.90 for the N36S/T42A/T66A mutant. These levels were 7- to 15-fold higher than the average L value of the low level CAM receptors.

In order to discuss our experimental data further, we implemented the two-state receptor model in the software program MatLab and composed a graphic interface to facilitate parameter input and curve simulation. We varied L values but fixed α and K_A at 0.2 and $5*10^{-8}$ M, respectively to see whether we could mimic our experimental data. When L values of 0.2, 2 and 200 were used, the overall shape of the simulated curves mimicked the curves that we obtained with receptors with varying levels of constitutive

activity (Fig. 4). The constitutive activity of CAM receptors with low L values (L = 0.2) was completely inhibited by the inverse agonist; CAM receptors with medium L values (L = 2) were partially inhibited (around 50%); and CAM receptors with high L values (L = 200) could not be inhibited by inverse agonists with a lowest α value of 0.2. Careful analysis of the simulated curves at L = 0.2 reveals that a slight amount of residual activity is expected to remain upon application of saturating levels of inverse agonists with an α value of 0.2. Apparently, this residual growth of the yeast cells is beyond the detection limit.

Besides these three types of simulated curves that we have also observed in our experiments, there was another type of curve that was not represented among our series of CAM receptors. When L is 20, the receptor could be inhibited by 15% by an inverse agonist with an α value of 0.2. We did not observe this level of inhibition in our experiments, because none of our CAM adenosine A_{2B} receptors displayed an L value of around 20.

Discussion

Evidence of constitutively active GPCRs has accumulated over the past 10 years, which enabled the distinction between neutral antagonists and inverse agonists for several GPCRs (Costa and Cotecchia, 2005). However, there is no report on constitutive activity for the wt human adenosine A_{2B} receptor. One might speculate that a high basal activity would disturb the physiological function of the adenosine A_{2B} receptor due to its ubiquitous expression (Volpini 2003). A possibly low constitutive activity of the wt adenosine A_{2B} receptor, however, would render discrimination between antagonists and inverse agonists for this receptor subtype difficult.

In our previous work a random mutation bank was constructed and a collection of adenosine A_{2B} receptor mutants with varying levels of constitutive activity was identified with a yeast growth assay (Beukers et al., 2004b). Yeast cells enabled us to identify CAM receptors among the randomly mutated receptors and these CAM receptors make it possible to study inverse agonism on the adenosine A_{2B} receptor. In this study, 9 mutant receptors with different levels of constitutive activity were used to examine inverse agonistic properties of 3 structurally different compounds, ZM241385, DPCPX and MRS1706. All three compounds have been described before in the literature as antagonists for the wild-type adenosine A_{2B} receptor (Alexander et al., 1996; Cooper et al., 1997; Pelletier et al., 2000; Poucher et al., 1995; Prentice et al., 1997; Ongini et al., 1999).

Before characterizing these three compounds on CAM A_{2B} receptors, we tested them on the wild-type human adenosine A_{2B} receptor expressed in yeast to check whether our yeast assay is comparable to assays with mammalian cells. The three compounds were

tested as antagonists by their ability to shift NECA-induced dose-growth curves and the data were compared with literature values. In our yeast assay, ZM241385 was able to antagonize NECA-induced yeast growth with a pA_2 value of 7.32±0.29, while for DPCPX a pA_2 value of 6.41±0.16 was calculated. As demonstrated in Table 2, the former was similar to the pA_2 value of 7.20 which was observed for antagonist ZM241385 to antagonize the A_{2B} receptor-mediated relaxant effect of NECA in the rat mesenteric artery (Prentice et al., 1997). The latter was close to the pA_2 value of 6.51 reported in guinea-pig tracheal epithelial cells for the ability of DPCPX to antagonize NECA-evoked cyclic AMP generation (Pelletier et al., 2000). No pA_2 value has been reported for MRS1706, but the pK_i value found in radioligand binding studies (8.86) confirms our findings that MRS1706 is a potent antagonist (Kim et al., 2000). As shown in Table 2, comparison of these literature data to our own results indicated that the potencies obtained in the yeast system were in good agreement with mammalian data. In both cell systems the rank of order of potency of MRS1706, ZM241385 and DPCPX on the wild-type adenosine receptor is MRS1706 ≥ ZM241385 > DPCPX.

Subsequent experiments on CAM adenosine A_{2B} receptors expressed in yeast provided firm evidence that these three compounds should be classified as inverse agonists rather than antagonists because they inhibited the growth of CAM receptors. Interestingly, all three structurally diverse inverse agonists tested in this study behaved in a similar manner, that is they all showed full inverse agonism on low level CAM receptors, partial inverse agonism on medium level CAM mutants and no significant inverse agonism on high level CAM mutants although they bound these CAM receptors as well as other CAM receptors in radioligand binding assays (data not shown).

To interpret the different intrinsic activities of the inverse agonists on the CAM receptors at the molecular level, the two-state receptor model was used. In this simplified model, two states are identified: an inactive state (here designated as R_i) and an active state (R_a). The equilibrium between receptors in the R_i versus R_a state may be altered by ligands (Lefkowitz et al., 1993; Leff, 1995; Kenakin, 1996). Agonists stabilize R_a while the inverse agonists stabilize the R_i state, and neutral antagonists have no preference for either state.

Simulation of our experimental data with the simple two state model revealed that the ability of inverse agonists to inhibit constitutively active receptors depends on the relative intrinsic efficacy (α) of the compounds as well as on the level of constitutive activity of the CAM receptor. Thus, low (L = 0.2) and medium level CAM receptors (L = 2) could be fully and partially inhibited by inverse agonists with α= 0.2, respectively. Stronger inverse agonists, with smaller α values, should in theory be able to fully inhibit not only these medium level CAM receptors but also the two CAM receptors that appear to be locked in an active state.

Two precedents for such “locked-on” receptors have been described in literature. For the adenosine A_1 receptor, we identified a locked mutant receptor containing a G14T mutation. Indeed this mutation led not only to constitutive activity but also to a "locked" phenotype in the sense that the basal activity of this receptor could not be modulated by either agonists or inverse agonists in both a GTPγS and a cAMP assay (de Ligt et al., 2005). In another report, accumulation of [^{3}H]IP in cells expressing Y368N mutant $5HT_{2C}$ receptors revealed that this mutant was also "locked" because none of the tested inverse agonists could inhibit the signaling of this receptor whereas these compounds

were able to inhibit the constitutive activity of the wild-type receptor and of another mutant receptor (Prioleau et al., 2002).

For inverse agonists, the smaller their α values are, the stronger inverse agonism they will exhibit. In the two-state model, the α value is a property that is intrinsic to a given compound provided that the intervening forcing function is the same within the test system. We therefore refer to this value as the relative intrinsic efficacy. In contrast, the intrinsic activity may vary for example when a mutation alters the interaction between receptor and G protein. Such a direct effect of a mutation on the intrinsic activity was described by Ganguli et al. on CAM secretin receptors (Ganguli et al., 1998). Three CAM secretin receptors with comparable levels of constitutive activity were created. Whereas the natural hormone secretin was able to activate the two single point mutants H156R and T322P, secretin turned out to be an inverse agonist on the double mutant receptor containing both mutations. In this case, the double mutant affected the intrinsic activity due to a reduction of the basal coupling of the receptor with G_s proteins.

In our study, on the other hand, the various CAM receptors display 3 different levels of constitutive activity (reflected in parameter L). The variation in intrinsic activity could be explained by these different L values as demonstrated by the simulations with variable L values whereas the relative intrinsic efficacy (α) and K_A values of the ligands were fixed. Mutants with the same level of constitutive activity behaved similarly to the same compound. This suggests that the mutated amino acids in the low and medium level CAM receptors are not part of the ligand binding but rather disturb the equilibrium between the active and inactive state of the receptor. In that case each individual compound will have the same dissociation constant (K_A) for the R_i state of the receptor,

which we observed indeed. In a similar vein each individual compound has the same preference for R_i or R_a state receptors, so its α value should also be identical. These considerations led us to the assumption that a compound has the same α value and K_A value on different mutants, which was the basis for our fitting method. It goes without saying that receptors with a mutation in the binding site (and hence a change in K_A value) cannot be subjected to this method.

Our simplified pharmacological model does not take into consideration the possibly multiple conformations of receptors in each state, receptor reserve or receptor to G protein-coupling. This two-state model assumes that no intervening forcing function is present. In the supplemental information to this paper the effect of such an intervening function, e.g. differences in coupling to G proteins or any other signal amplification effect is demonstrated. Extension of the two state model with such an intervening forcing function will alter the proportion of active receptors (ρ) and as a result will yield the 'true' efficacy value rather than the relative intrinsic efficacy value. Thus although we are able to model our data with the simplified two-state model we cannot rule out that different mutants display different levels of amplification effects.

With this model we were able to simulate our experimental observations, shedding light on the efficacy of inverse agonists, an issue not studied before. The availability of mutant receptors with varying levels of constitutive activity enabled us to determine the rank order of relative intrinsic efficacy values for different inverse agonists.

Based on the experimental data, the rank order of relative intrinsic efficacy of ZM241385, DPCPX and MRS1706 is ZM241385>MRS1706≈DPCPX. In other words, despite its higher potency, MRS1706 is a weaker inverse agonist than ZM241385.

JPET Fast Forward. Published on October 31, 2006 as DOI: 10.1124/jpet.106.111203
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In conclusion, we were able to characterize in a yeast growth assay ZM241385, DPCPX and MRS1706 as inverse agonists on the human adenosine A_{2B} receptor. The investigations allowed us to study the effects of inverse agonists on receptors with different levels of constitutive activity, which to our knowledge is the first study of such nature. We learned that mutated adenosine A_{2B} receptors with different levels of constitutive activity responded differently to three inverse agonists. Two high level CAM receptors were locked in an active state and were insensitive to the inverse agonists tested. The three intermediate level CAM receptors were partially inhibited, whereas the four low level CAM receptors were almost completely inhibited. These differences can be explained with different isomerization constant (L) values according to the two-state receptor model.

Acknowledgements

We should like to express our gratitude to Dr. T. Kenakin for discussing our findings and providing us with the simulation data provided in the supplemental data section. In it the two-state model is compared with an adapted two-state model that considers intervening functions.

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Legends for Figures

Fig. 1 Concentration-response curves of NECA in the absence or presence of 10 μM, 1 μM and 0.1 μM ZM241385 (a), 100 μM, 20 μM and 2 μM DPCPX (b), and 5 μM, 0.5μM and 0.1 μM MRS1706 (c). Schild plot analyses for the determination of pA_2 values of ZM241385, DPCPX, and MRS1706 at the adenosine A_{2B} receptor are shown as inserts.

Fig. 2 Concentration-response curves of ZM241385, DPCPX, and MRS1706-induced inhibition of yeast growth. Yeast cells expressing 9 CAM adenosine A_{2B} receptors with different levels of constitutve activity were tested in histidine-deficient solid medium containing 7 mM 3AT and concentrations of ZM241385 (a), DPCPX (b) or MRS1706 (c) as indicated. Growth of the yeast cells was scanned and quantified with Quantity One imaging software. One representative experiment performed in duplicate is shown of at least three independent experiments.

Fig. 3 Effect of ZM241385 on the constitutive activity of the highly constitutively active A18T/A23V/C83Y/A106V/R112S (■) and Q214L/I230N/V240M/V250M/N254Y/T257S/K269stop (▼) adenosine A_{2B} receptors. No significant inhibition of yeast growth was observed after treatment with ZM241385.

Fig. 4 Simulated curves versus experimentally determined curves. a) Simulated dose-proportion (ρ) curves. The parameters were: L = 0.2 (mixed line), 2 (continuous line), 20 (dotted line) and 200 (dashed line); α = 0.2; K_A = 50 nM. b) Experimentally determined dose-proportion (ρ) curves from growth assays of mutants F84L (●), N36S/T42A (▲) and Q214L/I230N/V240M/V250M/N254Y/T257S/K269stop (▼) on agar plates containing a range of concentrations of the inverse agonist MRS1706. On the X-axis the logarithm of the concentration of the ligand is shown. The Y-axis in a) describes the portion of receptors in the active state while the Y-axis in b) reflects the portion of receptors in the active state as calculated by the ratio of the growth of

yeast cells expressing various CAM receptors versus the highest amount of growth obtained in yeast cells expressing a locked receptor.

JPET Fast Forward. Published on October 31, 2006 as DOI: 10.1124/jpet.106.111203
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Table 1. Constitutive activity of wild-type and mutant human adenosine A_{2B} receptors.

Yeast cells expressing the wild-type or one of the 9 mutant adenosine A_{2B} receptors were screened on selection plates containing minimal agar medium plus tryptophan, adenine and 7 mM 3AT, the latter to suppress receptor-independent growth. "-" indicates that yeast cells did not grow on the selection plate due to the lack of constitutive receptor activity; "+" indicates that yeast cells did grow on the selection plates but only a weak constitutive activity was detectable; "++" indicates that the mutants exhibited constitutive activity 2 to 4 fold higher than "+"; "+++" indicates that the constitutive activity of the mutants was at least 5 fold greater than "+".

WT/Mutants	Constitutive activity
Wild-type	-
T42A	+
F84L	+
F84S	+
F84L/S95G	+
N36S/T42A	++
N36S/T42A/T66A	++
T42A/V54A	++
A18T/A23V/C83Y/A106V/R112S	+++
Q214L/I230N/V240M/V250M/N254Y/T257S/K269stop	+++

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Table 2. Comparison of the pA_2 values of ZM241385, DPCPX and MRS1706 as determined through antagonism of NECA-induced activation of adenosine A_{2B} receptors in this study versus literature data obtained in different tissues and cells.

Compound	Tissue or cells	pA_2 from literature	pA_2 this study
ZM241385	Rat mesenteric artery[a]	7.20 ± 0.12	7.32 ± 0.29
	CHO cells[b]	7.32 (7.17-7.48)	
DPCPX	HEK293[c]	7.01	
	CHO.A2B4 cell[d]	7.16	6.41 ± 0.16
	Guinea-pig cerebral cortex[e]	6.91	
	Guinea-pig tracheal epithelial cells[f]	6.51 ± 0.29	
MRS1706	n. d.		7.38 ± 0.18

[a]. Prentice et al., 1997

[b]. Ongini et al., 1999

[c]. Cooper et al., 1997

[d]. Alexander et al., 1996

[e]. Poucher et al., 1995

[f]. Pelletier et al., 2000

n. d. = not determined

Table 3. Comparison of the constitutive activity (CA), potency (IC_{50} value) , intrinsic activity (% inhibition) and relative efficacy (R.E.) of ZM241385, DPCPX and MRS1706 towards CAM adenosine A_{2B} receptors with low and intermediate levels of constitutive activity.

Mutants	CA	ZM241385			DPCPX			MRS1706		
		IC_{50} (nM)	I_{max} (%)	R.E(%)	IC_{50} (μM)	I_{max} (%)	R.E.(%)	IC_{50} (nM)	I_{max} (%)	R.E.(%)
F84L	+	135±88	95±7	100	1.44±1.39	92±3	97	43±21	91±12	96
F84S	+	107±37	99±2	100	1.91±0.68	97±3	98	54±12	94±6	95
F84L/S95G	+	102±60	97±4	100	1.43±0.31	99±2	102	40±32	94±8	97
T42A	+	71±34	96±5	100	1.55±0.98	96±2	100	98±62	93±5	97
T42A/V54A	++	210±86	79±7	100	0.86±0.31	40±12	51	166±138	54±5	68
N36S/T42A	++	187±105	74±7	100	1.44±1.02	58±3	78	133±66	57±11	77
N36S/T42A/T66A	++	522±239	63±4	100	6.25±2.46	39±11	62	[a]	28±16	44

IC_{50}, I_{max} are expressed as mean ± S.E.M. from at least 3 independent experiments. Relative efficacy was the ratio (%) of maximum inhibition induced by an inverse agonist divided by that of full inverse agonist ZM241385. [a] IC_{50} value could not be determined due to small experimental window.

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Table 4 Fitted values of the L parameter for mutants with low level or medium level constitutive activity

mutants	ZM241385	DPCPX	MRS1706
F84L	0.11±0.02	0.12±0.02	0.14±0.02
F84S	0.22±0.03	0.16±0.02	0.22±0.02
F84L/S95G	0.22±0.02	0.17±0.02	0.20±0.02
T42A	0.20±0.02	0.16±0.02	0.18±0.02
T42A/V54A	1.38±0.10	1.6±0.10	1.38±0.08
N36S/T42A	1.49±0.10	1.47±0.10	1.52±0.08
N36S/T42A/T66A	1.9±0.17	1.69±0.10	1.90±0.11

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Figure 1

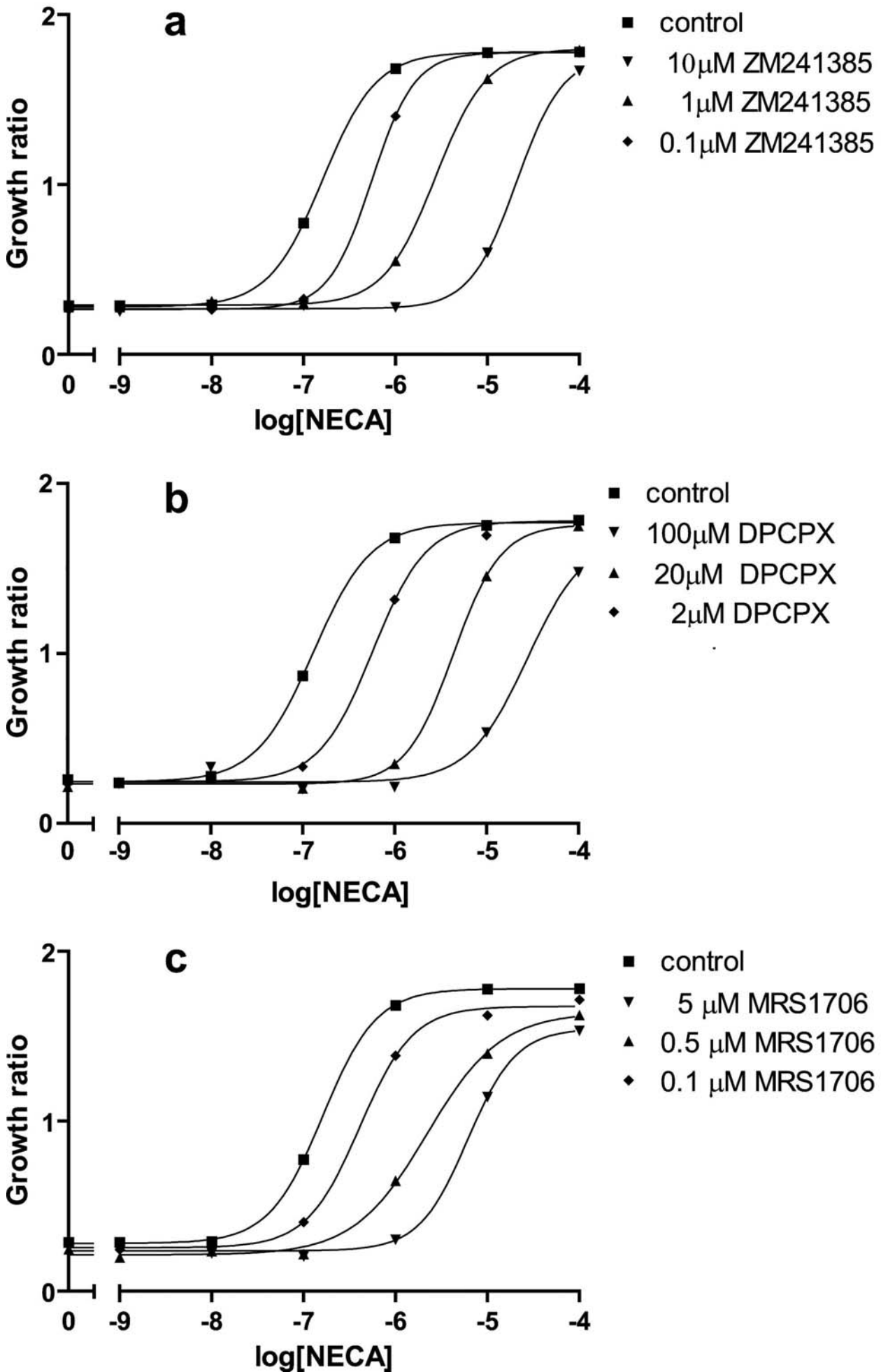

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Figure 2

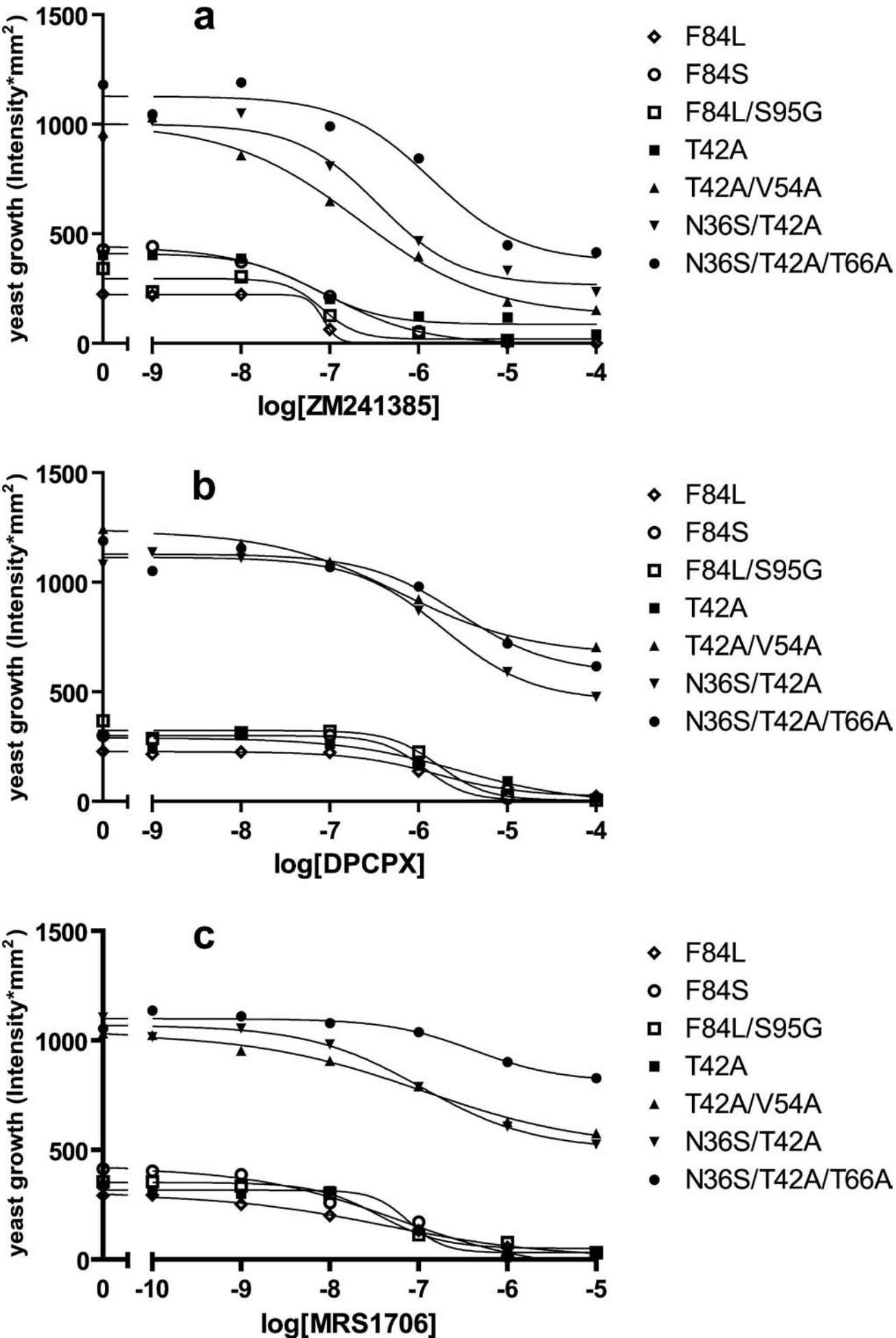

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Figure 3

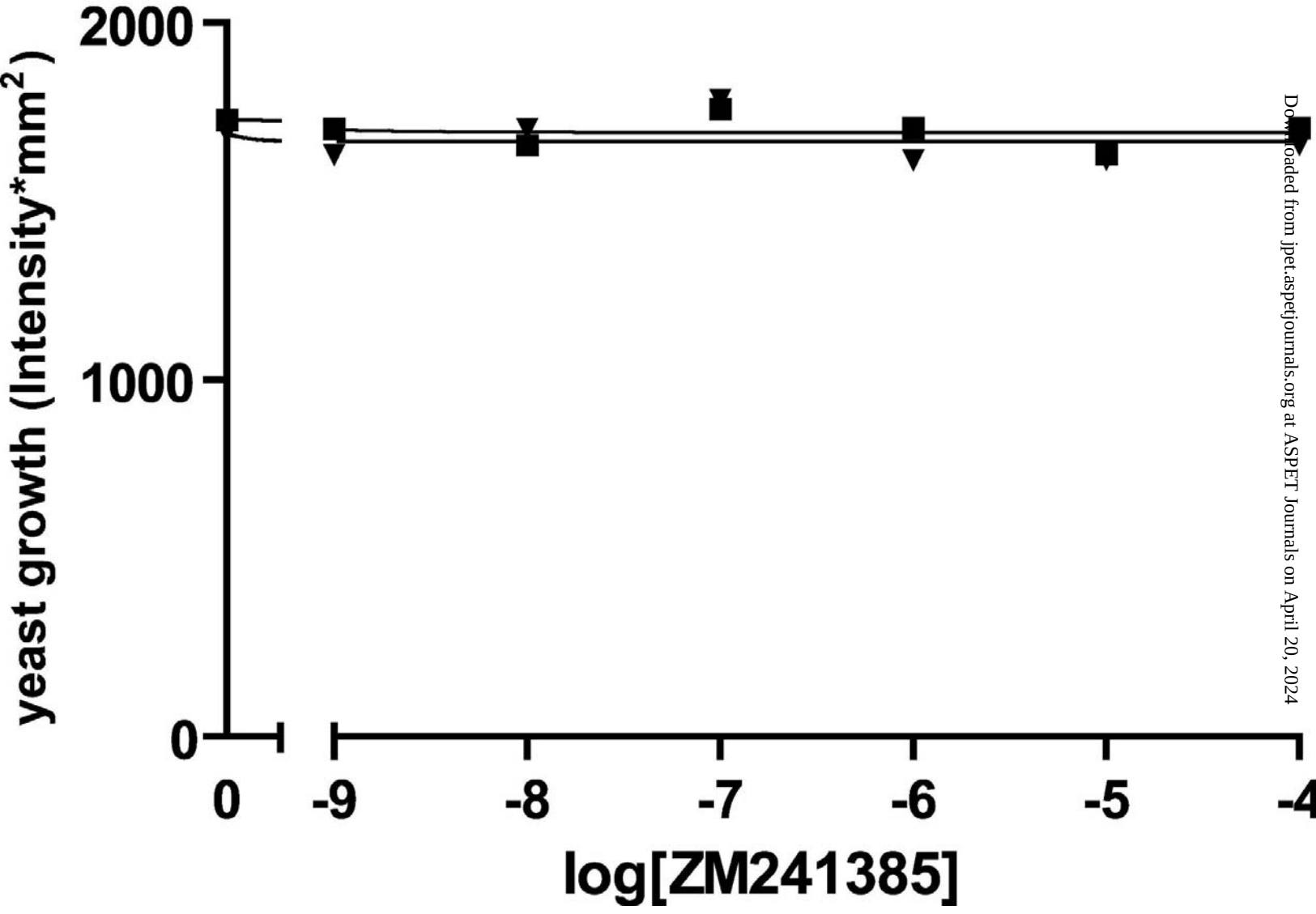

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Figure 4