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Inhibition of Receptor/G Protein Coupling by Suramin Analogues

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SUMMARY

Suramin analogues act as direct antagonists of heterotrimeric G proteins because they block the rate-limiting step of G protein activation (i.e., the dissociation of GDP prebound to the G protein α subunit). We have used the human brain A_1 adenosine receptor and the rat striatal D_2 dopamine receptor, two prototypical G_i/G_o-coupled receptors, as a model system to test whether the following analogues suppress the receptor-dependent activation of G proteins: 8-(3-nitrobenzamido)-1,3,5-naphthalenetrisulfonic acid (NF007), 8-(3-(3-nitrobenzamido)-benzamido)-1,3,5-naphthalenetrisulfonic acid (NF018); 8,8′-(carbonylbis(imino-3,1-phenylene))bis-(1,3,5-naphthalenetrisulfonic acid) (NF023); 8,8′-(carbonylbis(imino-3,1-phenylene)carbonylimino-(3,1-phenylene))bis-(1,3,5-naphthalenetrisulfonic acid) (NF037); and suramin. Suramin and its analogues inhibit the formation of the agonist-specific ternary complex (agonist/receptor/G protein). This inhibition is (i) quasicompetitive with respect to agonist binding in that it can be overcome by increasing receptor occupancy but (ii) does not result from an interaction of the analogues with the ligand binding pocket of the receptors because the binding of antagonists or of agonists in the absence of functional receptor/G protein interaction is not affected. In addition to suppressing the spontaneous release of GDP from defined G protein α subunits, suramin and its analogues reduce receptor-catalyzed guanine nucleotide exchange. The site, to which suramin analogues bind, overlaps with the docking site for the receptor on the G protein α subunit. The structure-activity relationships for inhibition of agonist binding to the A_1 adenosine receptor (suramin > NF037 > NF023) and of agonist binding to the inhibition D_2 dopamine receptor (suramin = NF037 > NF023 > NF018) differ. Thus, NF037 discriminates between the ternary complexes formed by the agonist-liganded D_2 dopamine receptors and those formed by the A_1 adenosine receptor with >10-fold selectivity. Therefore, our results also show that inhibitors can be identified that selectively uncouple specific receptor/G protein tandems.

The G protein subunits display a large degree of molecular diversity. Currently, >20 individual G protein α subunits are known. In addition, there are five G protein β subunits and more than seven γ subunits. The biological significance of this diversity is not fully understood. A set of experimental strategies has been used to assess the specificity of interactions that govern coupling of the G protein to the receptor or to its cellular effector molecules (for reviews, see Refs. 1–3). These experiments have established that effectors do not distinguish between individual closely related G protein α subunits or $\beta\gamma$ dimers (4–7) but that some receptors are capable of discriminating between related G protein α subunits (8–12) and $\beta\gamma$ dimers of defined composition (13, 14). This selectivity of receptor/G protein coupling has been substantiated by using antisense oligonucleotides to specifically deplete individual subunits in intact cells (15–17). This strategy has highlighted *in vivo* that a stringent requirement of a given receptor exists for a G protein oligomer of defined subunit composition to elicit a biological response.

Given this high degree of specificity in the interaction between receptor and G protein and the large variety of G protein oligomers that can be produced by combinatorial association, we believe that the receptor binding sites of G protein oligomers *per se* should be considered as potential drug targets. Drugs interfering with this site will specifically act downstream of the initial signaling event (interaction between receptor ligands and receptor) and block the effect of the receptor. In addition to being selective for individual G

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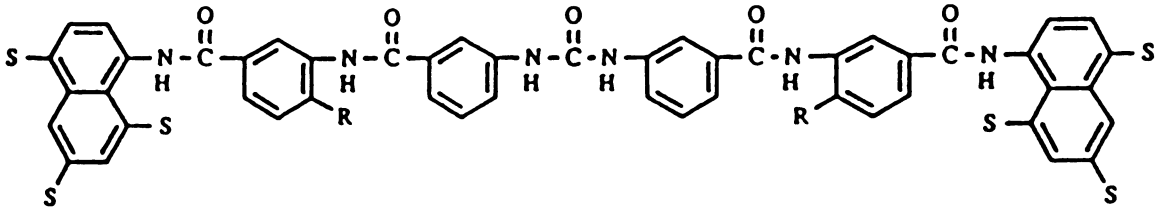
ABBREVIATIONS: NF007, 8-(3-nitrobenzamido)-1,3,5-naphthalenetrisulfonic acid; NF018, 8-(3-(3-nitrobenzamido)benzamido)-1,3,5-naphthalenetrisulfonic acid; NF023, 8,8′-(carbonylbis(imino-3,1-phenylene))bis-(1,3,5-naphthalenetrisulfonic acid); NF037, 8,8′-(carbonylbis(imino-3,1-phenylene)carbonylimino-(3,1-phenylene))bis-(1,3,5-naphthalenetrisulfonic acid); HPIA, N^6-(4-hydroxyphenylisopropyl)adenosine; CPA, N^6-cyclopentyladenosine; XAC, xanthine amine congener; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine 5′-(3-O-thio)triphosphate

TABLE 1

Inhibition of receptor-G protein coupling in brain membranes: affinity estimates for suramin analogues

Estimates (mean ± standard deviation, three experiments) for the concentration at which half-maximal inhibition of agonist binding (IC_{50}) or half-maximal stimulation of antagonist binding occurs (EC_{50}) was calculated by fitting the data summarized in Figs. 5A and 8 to a three-parameter logistic equation.

	A_1-adenosine receptor agonist binding IC_{50}	D_2-dopamine receptor agonist binding IC_{50}	D_2-dopamine receptor antagonist binding EC_{50}
	μM	μM	μM
NF023	66 ± 11	8.8 ± 1.2	3.5 ± 0.6
NF037	34 ± 5	1.6 ± 0.3	0.6 ± 0.1
Suramin	3.1 ± 0.5	1.2 ± 0.2	0.5 ± 0.1



Structure of suramin (S=SO_3Na; R=CH3 in suramin and H in all analgoues)

NF037 is didemethylated suramin; NF023 corresponds to NF037, lacking two benzamide rings on each side of the central urea bridge; and NF007 and NF018 are "half-molecules" of NF023 and NF037, respectively.

proteins, these compounds may display a new type of selectivity by discriminating between individual receptor/G protein complexes. Previously, we characterized the interaction of suramin analogues with G protein α subunits. These compounds directly decrease the basal rate of guanine nucleotide exchange, an effect that can be reversed by the addition of the effector (18). In the current study, we investigated whether suramin and its analogues impede the interaction between receptor and G protein by using A_1 adenosine and D_2 dopamine receptors as model systems because the G protein specificity of these receptors has been well characterized (8, 10, 19).

Experimental Procedures

Materials. [^{35}S]GTPγS, [^{3}H]DPCPX, and ^{125}I were purchased from New England Nuclear (Boston, MA); [^{125}I]epideprid was purchased from Research Center Seibersdorf (Seibersdorf, Austria); guanine nucleotides and adenosine deaminase were purchased from Boehringer Mannheim (Mannheim, Germany); DPCPX, XAC, and sulpiride were purchased from Resarch Biochemicals (Natick, MA); and apomorphine and CPA were purchased from Sigma Chemical Co. (St. Louis, MO). ^{125}I-HPIA was synthesized by radioiodination of HPIA and purified by HPLC according to Linden (20). Suramin was a generous gift from Bayer AG (Wuppertal, Germany), the synthesis of the suramin analogues used in the study (NF007, NF018, NF023, NF037) has been described previously (21). The structural formula of suramin is shown in Table 1.

Membrane preparation and protein purification. The source of human cerebral cortex brain and the preparation of human brain membranes have been described previously (19). The human A_1 adenosine receptor was expressed in *Escherichia coli* under the control of the maltose binding protein promoter, and bacterial membranes were prepared as outlined previously (19). For the preparation of rat striatal membranes, the animals were killed by decapitation, and the brains were removed and dissected. The striatal tissue was homogenized in 4 volumes of buffer containing 10 mM HEPES·NaOH, pH 7.4, 1 mM $MgCl_2$, and 250 mM sucrose with the use of a motor-driven, tight-fitting Teflon pestle (10 strokes). The homogenate was subjected to differential centrifugation (10 min at 9,000 × *g* followed by 10 min at 50,000 × *g*). The resulting pellet was washed free of sucrose; taken up at a protein concentration of 5 mg/ml in 20 mM HEPES·NaOH, pH 7.4, 2 mM $MgCl_2$, and 1 mM EDTA; quick frozen in liquid nitrogen; and stored at −80°. The protein concentration was determined by dye binding with the Bio-Rad Coomassie-Blue kit using bovine serum albumin as the standard.

Receptor binding assays. Binding experiments with the A_1 adenosine receptor antagonist radioligand [^{3}H]DPCPX were carried out in a final volume of 0.1 ml containing 50 mM Tris·HCl, pH 8, 1 mM EDTA, 5 mM $MgCl_2$, 8 mg/ml adenosine deaminase, and 50–150 μg of bacterial or human brain membrane protein, and the concentrations of [^{3}H]DPCPX, competitors, suramin, and GTPγS indicated in the figures. After 60 min at 20°, the reaction was stopped by filtration over glass-fiber filters. Nonspecific binding was determined in the presence of 10 μM CPA or 10 μM XAC and amounted to 5–15% of total binding in the K_D concentration range.

High affinity binding of the A_1 adenosine receptor agonist radioligand ^{125}I-HPIA to human brain membranes (10–30 μg) was determined in 50 μl containing 50 mM Tris·HCl, pH 8, 1 mM EDTA, 5 mM $MgCl_2$, 8 mg/ml adenosine deaminase, and the concentrations of ^{125}I-HPIA, competitors, GTPγS, and suramin analogues indicated in the figures. The reaction was carried out for 90 min at 20° and stopped by filtration over glass-fiber filters. In kinetic experiments, the binding was allowed to reach equilibrium (60 min at 20° and ~1 nM ^{125}I-HPIA); thereafter, competitors, suramin analogues, and GTPγS were added. Aliquots (30 μl containing 10–20 μg of membrane protein) were withdrawn, and the reaction was stopped at the time points indicated in the figures.

Binding experiments with the D_2 dopamine receptor antagonist radioligand [^{125}I]epideprid were carried out in 0.1 ml containing 50 mM Tris·HCl, pH 8, 1 mM EDTA, 5 mM $MgCl_2$, rat striatal membranes (7.5–15 μg), and the concentrations of [^{125}I]epideprid, competitors, suramin, and GTPγS indicated in the figures. After 60 min at 20°, the reaction was stopped by filtration over glass-fiber filters. Nonspecific binding determined in the presence of 10 μM sulpiride amounted to ~5% of total binding in the K_D concentration range.

GTPγS-binding assay. Receptor-promoted binding of [^{35}S]GTPγS to human brain membranes was determined as described (22); briefly, membranes (~15 μg) were suspended in 40 μl buffer containing 20 mM HEPES·NaOH, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM GDP, and the concentrations of CPA, DPCPX, and suramin analogues indicated in the figures. After a preincubation of 10 min at 20°, 10 μl of buffer containing [^{35}S]GTPγS was added to yield a final concentration of 5 nM (specific activity, 2000 cpm/fmol). The reaction was stopped at the time points indicated with 1 ml of ice-cold stop buffer containing 10 mM Tris·HCl, pH

8, 100 mM NaCl, 20 mM $MgCl_2$, and 0.1 mM GTP. Bound and free nucleotides were separated by filtration over glass-fiber filters, which were rinsed with 20 ml of wash buffer (stop buffer without GTP).

If not stated otherwise, data are from representative experiments carried out in duplicate. Each experiment was carried out at least three times. The intra-assay and interassay variation was ≤5% and ≤15%, respectively. Data were subjected to nonlinear, least-squares curve fitting using the appropriate equations (rectangular hyperbola, Hill equation, monophasic exponential decay), and parameter estimates were derived.

Results

Inhibition of agonist binding to the A_1 adenosine receptor by suramin. The high affinity binding of agonists depends on the formation of the ternary complex HRG between agonist (H), receptor (R), and G protein (G) (1–3). Compounds that interfere with the coupling between receptor and G protein are thus expected to suppress agonist binding. We therefore investigated the effect of suramin on the high affinity binding of the agonist radioligand ^{125}I-HPIA to A_1 adenosine receptors in human brain membranes. When added to the incubation, suramin inhibits equilibrium binding of the agonist radioligand (Fig. 1C). The trivial explanation for this phenomenon is that suramin interacts with the ligand binding pocket of the A_1 adenosine receptor and thus blocks access of the radioligand (competitive antagonism). Alternatively, suramin may suppress the interaction between receptor and G protein by binding to the G protein α subunit and thus blocks the formation of the ternary complex (allosteric inhibition). We carried out three types of experiments to differentiate between these two possibilities: (i) inhibition of agonist binding at two different radioligand concentrations (Fig. 1), (ii) saturation and competition experiments with the radiolabeled A_1 adenosine receptor antagonist [^{3}H]DPCPX (Figs. 2 and 3), and (iii) destabilization of the preformed high affinity complex (Fig. 4).

In radioligand binding experiments, the effect of allosteric inhibitors should be independent of the radioligand concentration. The ability of suramin to inhibit the binding of ^{125}I-HPIA to human brain membranes, however, depended on the concentration of the radioligand used (Fig. 1C). The IC_{50} was shifted by a factor of 2.1-fold if the concentration of ^{125}I-HPIA was raised from 0.5 to 5 nM (IC_{50} = 3.9 ± 0.4 and 8.2 ± 0.9 μM, respectively; Hill coefficient n_H = 0.92 ± 0.10 and 1.06 ± 0.16 at 0.5 and 5 nM ^{125}I-HPIA, respectively; three experiments). A similar shift (2.2-fold; IC_{50} = 0.26 ± 0.06 and 0.57 ± 0.05 nM, respectively; n_H = 1.10 ± 0.09 and 1.13 ± 0.11 at 0.5 and 5 nM ^{125}I-HPIA, respectively; three experiments) was observed for the A_1-selective agonist CPA (Fig. 1A) and for the antagonist XAC (not shown). Guanine nucleotides inhibit high affinity agonist binding in an allosteric manner by destabilizing the ternary HRG complex. As expected, the apparent affinity of the hydrolysis-resistant GTP analogue GTPγS did not vary with the radioligand concentration (Fig. 1B; IC_{50} = 43.8 ± 5.6 and 44.3 ± 4.3 nM at 0.5 and 5 nM ^{125}I-HPIA, respectively; three experiments). According to the Cheng-Prusoff approximation [$K_i = IC_{50}/(1 + L/K_D)$], the IC_{50} of a competing ligand (the concentration that displaces 50% of the radioligand) depends on the radioligand concentration L in a linear manner and is determined inversely by both the dissociation constant K_D of the radioligand and that of the competing ligand K_i: $IC_{50} = K_i * (1 + L/K_D)$. The K_D for the radioligand ^{125}I-HPIA determined in saturation experiments carried out in parallel was 2 nM. A shift by a factor of ~2.8 is calculated for a competitive inhibitor if the experiments are carried out at 0.5 and 5 nM ^{125}I-HPIA and the observed shifts for CPA and suramin are in reasonable agreement with this value. Thus, based on these criteria, suramin seems to act as competitive inhibitor for ^{125}I-HPIA binding. The straightforward interpretation is that suramin interacts directly with the ligand binding pocket of the A_1 adenosine receptor.

This possibility, however, was clearly ruled out by the experiments summarized in Figs. 2 and 3. When added to

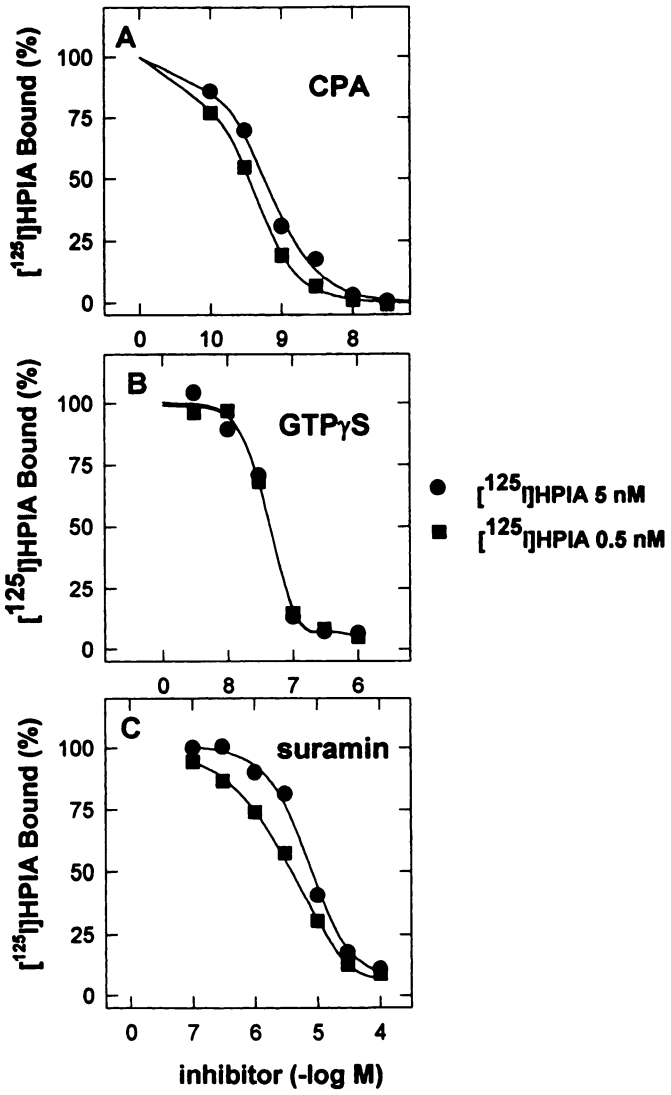


Fig. 1. Inhibition of equilibrium ^{125}I-HPIA binding to human cortical brain membranes by CPA (A), GTPγS (B), and suramin (C). High affinity ^{125}I-HPIA binding to human brain membranes (30 μg) was determined in a final volume of 50 μl containing 50 mM Tris·HCl, pH 8, 1 mM EDTA, 5 mM $MgCl_2$, 8 mg/ml adenosine deaminase, 0.5 nM (■) and 5 nM ^{125}I-HPIA (●), and the indicated concentrations of CPA, GTPγS, and suramin. The reaction was carried out for 90 min at 20°. Specific binding determined in the absence of any inhibitor amounted to 2 and 4.8 fmol/assay for 0.5 and 5 nM ^{125}I-HPIA, respectively, and was set at 100%. Nonspecific binding determined in the presence of 1 μM CPA was ~5 and ~20% of total binding at 0.5 and 5 nM ^{125}I-HPIA, respectively. Data are from a single experiment carried out in parallel, which is representative for two additional experiments.

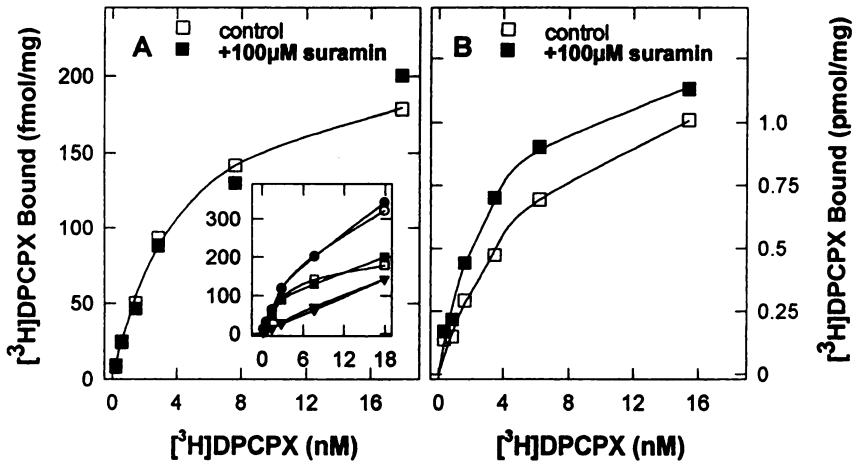


Fig. 2. Equilibrium [^{3}H]DPCPX binding to *E. coli* membranes expressing the human A_1 adenosine receptor (A) and human cortical brain membranes (B) in the absence and presence of suramin. Binding experiments with the antagonist radioligand [^{3}H]DPCPX were carried out in a final volume of 0.1 ml containing 50 mM Tris·HCl, pH 8, 1 mM EDTA, 5 mM $MgCl_2$, 8 mg/ml adenosine deaminase, bacterial (100 μg) or human brain membrane protein (50 μg), and the concentrations of [^{3}H]DPCPX indicated for 60 min at 20°. The specific binding in the absence (□) and presence of suramin (■) is shown. Nonspecific binding was determined in the presence of 10 μM XAC and amounted to 15% (A) and 5% (B) of total binding in the K_D concentration range. *Inset*, suramin affects neither total binding (○, ●) nor nonspecific binding (▽, ▼) in the bacterial membranes. Data are mean from duplicate determinations in a single experiment; two additional experiments gave similar results.

human brain membranes at a concentration of 100 μM, which has a profound inhibitory effect on the binding of the agonist radioligand ^{125}I-HPIA, suramin does not inhibit but rather enhances the binding of the antagonist [^{3}H]DPCPX (Fig. 2B). This modest stimulation of antagonist binding in human brain membranes is also compatible with the ternary complex model; antagonists bind preferentially to the G protein-free receptor (23–26). If suramin blocked receptor/G protein interaction, antagonist binding should increase in the presence of G proteins. We verified that suramin does not affect [^{3}H]DPCPX binding in the absence of G proteins (Fig. 2A) by using membranes prepared from *E. coli* expressing the human A_1 adenosine receptor (19). None of the suramin analogues used were found to inhibit antagonist binding up to a concentration of 100 μM (not shown). In G protein-coupled receptors, binding of agonists occurs via bonding with amino acid residues in the ligand binding pocket, which do not necessarily interact with the antagonist (27, 28). We therefore also tested the possibility that suramin may selectively affect the surface of the ligand binding pocket, which is required for agonist binding. We measured the ability of the agonist CPA to displace [^{3}H]DPCPX binding in the presence or absence of 100 μM suramin. The antagonist XAC was used as control (Fig. 3). Suramin did not affect the ability of the agonist CPA (and of the antagonist XAC) to compete for [^{3}H]DPCPX binding in *E. coli* membranes or human brain membranes in the presence of GTPγS (Fig. 3, A and B).

The results presented so far suggest that an effect of suramin can only be observed if the A_1 adenosine receptor is functionally coupled to G proteins. Additional evidence for this interpretation (i.e., that the site of action for suramin is at the interaction site between receptor and G protein) was obtained by determining the effect of suramin on the kinetics of radioligand dissociation (Fig. 4). The addition of both the agonist CPA and the antagonist XAC induced a slow dissociation of ^{125}I-HPIA with identical rate constants (0.010 ± 0.001 and 0.009 ± 0.002 min^{-1} for CPA and XAC, respectively; three experiments). In contrast, the addition of GTPγS results in instantaneous dissociation. This reaction reflects the very rapid destabilization of the ternary complex resulting from the formation of the activated GTPγS-liganded α subunit, which dissociates from the βγ subunit complex (1, 25). Similarly, suramin destabilized ^{125}I-HPIA binding; the observed dissociation rates were ~3-fold faster (0.027 ± 0.005 min^{-1}; three experiments) than those measured in the presence of the receptor ligands CPA and XAC. This observation strictly argues against direct interference with ligand binding. To account for an increase in the agonist dissociation rate, suramin must be able to impede the contact between receptor and G protein in the high affinity ternary complex.

Structure-activity relation for inhibition of A_1 adenosine receptor/G protein coupling by suramin analogues. We compared the ability of suramin analogues to inhibit ^{125}I-HPIA binding (Fig. 5A) and to destabilize preformed ternary complexes (Fig. 5B). Suramin was the most potent analogue, followed by NF037 and NF023 (Table 1). We also tested the ability of the analogues to suppress the agonist-dependent stimulation of GTPγS-binding to human brain membranes (Fig. 6). Under the experimental conditions used, the agonist CPA markedly stimulated GTPγS-binding so that the A_1 adenosine receptor-dependent binding accounted for 90% of the total binding. The receptor-promoted exchange reaction was linear for ≤10 min; NF023 inhibited the receptor-dependent stimulation of [^{35}S]GTPγS-binding, whereas the receptor-independent component was barely affected (Fig. 6A). This was also observed for the other suramin analogues (not shown). As can be seen from the data summarized in Fig. 6B, the structure-activity relationship for the inhibition of CPA-stimulated GTPγS-binding was suramin > NF037 > NF023. This rank order is identical to that observed for inhibition of high affinity agonist binding or destabilization of the preformed A_1 adenosine receptor/G protein complex (see Fig. 5, A and B).

Inhibition of agonist binding to the D_2 dopamine receptor by suramin. We assessed the ability of suramin analogues to inhibit the interaction between the D_2 dopamine receptor, another prototypical G_i/G_o-coupled receptor (G_i is the G protein mediating inhibition of adenylyl cyclase;

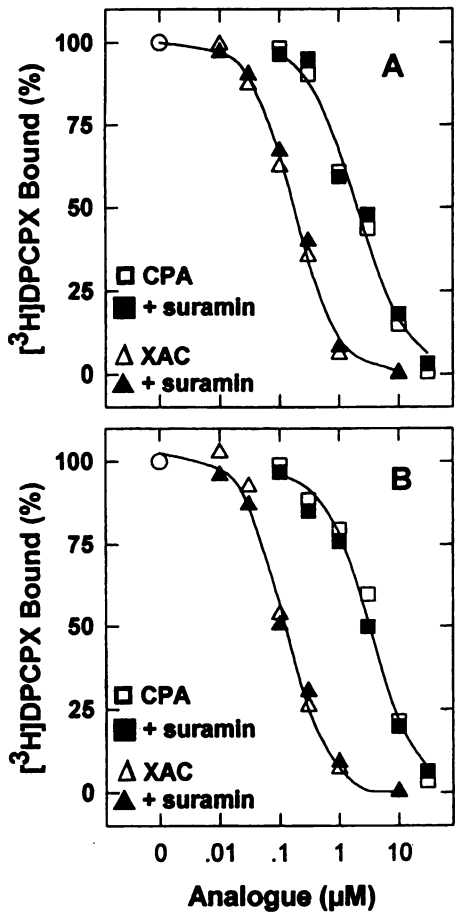


Fig. 3. Competition by CPA and XAC for [^{3}H]DPCPX binding to *E. coli* membranes expressing the human A_1 adenosine receptor (A) and human cortical brain membranes (B) in the absence and presence of suramin. The binding reaction was carried out with 3 nM [^{3}H]DPCPX in 0.1 ml containing bacterial (150 μg) or human brain membrane protein (50 μg) and CPA (□, ■) or XAC (△, ▲) in the absence (□, △) and presence (■, ▲) of suramin. Incubations carried out with human brain membranes contained GTPγS (100 μM). Reaction conditions were as outlined in the legend to Fig. 2. Data are mean from duplicate determinations in a single experiment; two additional experiments gave similar results.

G_o is the G protein involved in the regulation of neuronal calcium channels), and endogenous G proteins. Suramin did not inhibit but rather enhanced binding of the antagonist [^{125}I]epideprid to rat striatal membranes (Fig. 7A). The addition of GTPγS enhanced antagonist binding to a comparable extent (not shown). Similar to the findings obtained with the A_1 adenosine receptor (see Fig. 2), this observation can thus be interpreted as a suramin-induced uncoupling of the D_2 dopamine receptor from G proteins resulting in an apparent increase in receptors that bind the antagonist with high affinity. As expected for a compound capable of impeding the interaction between D_2 dopamine receptor and G proteins, suramin decreased the ability of the agonist apomorphine to displace [^{125}I]epideprid by shifting the agonist competition curve to the right (Fig. 7B, ■). The shift was concentration dependent (data not shown), and the effect of maximally active concentrations was comparable to that of GTPγS, which also shifts the agonist competition curve to the right (Fig. 7B, ▲). The combination of suramin and GTPγS at maximally active concentrations did not produce an additional effect (Fig. 7B, ▼).

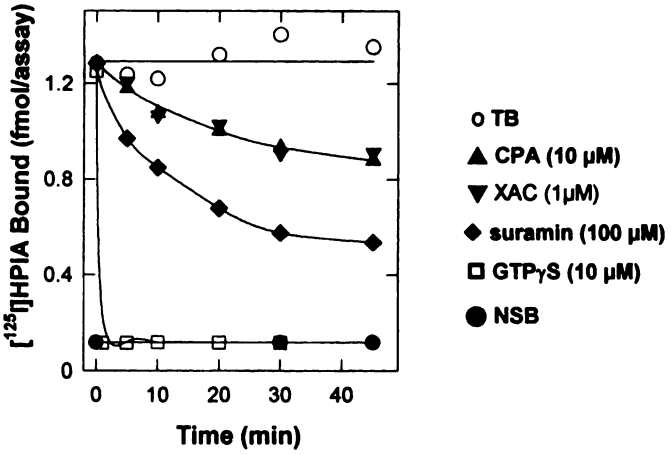


Fig. 4. Dissociation by CPA, XAC, suramin, and GTPγS of equilibrium ^{125}I-HPIA binding to human cortical brain membranes. Binding of ^{125}I-HPIA (1 nM) to human brain membranes was allowed to reach equilibrium (60 min at 20°) under the conditions outlined in the legend to Fig. 1; thereafter, CPA (▲), XAC (▼), GTPγS (□), and suramin (◆) were added to a final concentration of 10 μM (CPA, XAC, GTPγS) or 100 μM (suramin). The control total binding (○, *TB*) and nonspecific binding in the presence of 1 μM CPA (●, *NSB*) were determined in parallel incubations. Aliquots (30 μl containing 10 μg of membrane protein) were withdrawn at the indicated time points, and the reaction was stopped by filtration over glass-fiber filters. Data are mean from duplicate determinations in a single experiment; two additional experiments gave similar results.

Structure-activity relation for inhibition of D_2 dopamine receptor/G protein coupling by suramin analogues. We compared the ability of the suramin analogues to enhance [^{125}I]epideprid binding (Fig. 8A) and to reverse the displacement of radioligand binding by 10 nM apomorphine (Fig. 8B). The structure-activity relationships were identical (suramin = NF037 > NF023 > NF018) but differs clearly from that observed for inhibition of A_1 adenosine receptor/G protein coupling (suramin > NF037 > NF023 ≫ NF018; see Fig. 5 and Table 1). This difference cannot be accounted for by the different tissue sources; inhibition of ^{125}I-HPIA binding to rat striatal membranes gave results comparable to the results presented for the human cerebral A_1 adenosine receptor (data not shown). In addition, lower concentrations are required to promote [^{125}I]epideprid binding than are required for reversal of the apomorphine-induced displacement (see Table 1). This discrepancy, however, can be rationalized if the higher affinity of the agonist-liganded receptor (i.e., the species formed in the presence of apomorphine) for the G protein is taken into account.

Discussion

Our previous work showed that suramin analogues interacted directly with G protein α subunits (18). Here, we demonstrated that these compounds also impede the activation of G proteins by receptors. This conclusion is based on the following observations. (i) Suramin analogues suppress high affinity agonist binding in membranes to both the A_1 adenosine receptor and the D_2 dopamine receptor. A direct interaction of suramin analogues with the ligand binding pocket of the receptors can be ruled out because the compounds affect the binding of neither antagonists nor agonists to receptors in the absence of G proteins or under conditions in which the receptor is uncoupled from the G protein. (ii) The

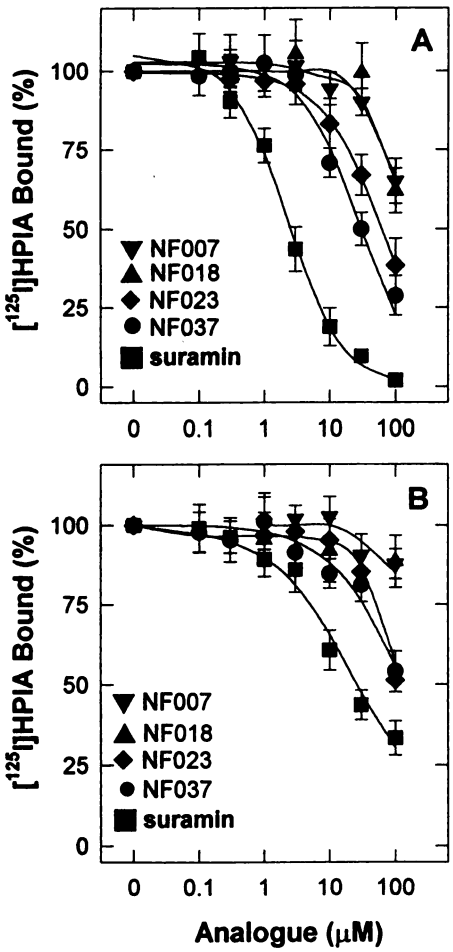


Fig. 5. Inhibition (A) and dissociation (B) of equilibrium ^{125}I-HPIA binding to human cortical brain membranes by suramin analogues. A, Equilibrium binding of ^{125}I-HPIA (0.5 nM) to membranes (20–30 μg) was determined in the absence (100%) and presence of suramin (■), NF007 (▼), NF018 (▲]), NF023 (◆), and NF037 (●) as outlined in the legend to Fig. 1. B, After 60 min of preincubation, dissociation of equilibrium binding of ^{125}I-HPIA (1 nM) to membranes (15 μg) was initiated by the addition of suramin (■), NF007 (▼), NF018 (▲), NF023 (◆), and NF037 (●) to yield the indicated final concentrations. The control binding (100%) was determined in the absence of any analogue. The incubation was continued for an additional 40 min. Assay conditions were as described in the legend to Figs. 1 and 4. Data are mean from three independent experiments carried out in duplicate. *Error bars*, standard deviation.

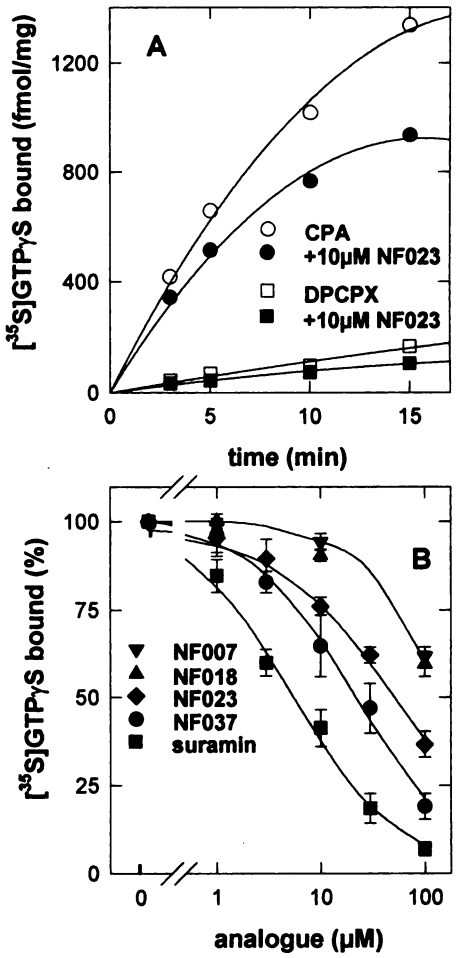


Fig. 6. Inhibition of the A_1 adenosine receptor agonist-mediated stimulation of [^{35}S]GTPγS binding to human cortical brain membranes by suramin analogues. A, Binding of [^{35}S]GTPγS to membranes (15 μg) was determined in 40 μl containing 20 mM HEPES·NaOH, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM GDP, and 12.5 μM CPA (○, ●) or 12.5 μM DPCPX (□, ■) in the absence (○, □) and presence (●, ■) of 12.5 μM NF023. After a 10-min preincubation at 20°, 10 μl of [^{35}S]GTPγS was added (5 nM final concentration, specific activity 2000 cpm/fmol); the reaction was stopped at the time points indicated. Data are mean from duplicate determinations in a single experiment; two additional experiments gave similar results. B, The ability of suramin (■), NF007 (▼), NF018 (▲), NF023 (◆), and NF037 (●) to inhibit the CPA-induced stimulation of [^{35}S]GTPγS binding was determined as outlined for A after an incubation time of 7.5 min. Binding in the absence of any suramin analogue was set at 100% and amounted to 725 ± 54 fmol/mg. Data are mean from three independent experiments carried out in duplicate. *Error bars*, standard error.

stimulation of guanine nucleotide exchange by the agonist-activated A_1 adenosine receptor is inhibited by suramin. (iii) Suramin analogues destabilize the preformed ternary complex of agonist radioligand, A_1 adenosine receptor, and G protein.

The productive interaction between receptor and G proteins is required to detect an effect of suramin analogues on agonist and antagonist binding. When tested in the presence of a concentration of ^{125}I-HPIA below and above the K_D of this radioligand, the apparent affinity of suramin was shifted in a manner consistent with competitive inhibition. However, a similar type of quasicompetitive inhibition is expected if the agonist-liganded receptor competes with suramin for binding to the G protein. Increasing the number of active receptors in the membrane by increasing agonist occupancy can overcome the suramin-dependent inhibition and thereby shift the suramin concentration curve to the right. In this situation, the Cheng-Prusoff analysis cannot provide a clue to differentiate between true competition for the ligand binding pocket and quasicompetitive inhibition by binding to the G protein. Two additional findings support this interpretation. (i) Formation of the ternary complex of ^{125}I-HPIA, adenosine receptor, and G protein is more sensitive to the inhibitory effect of suramin and its analogues than is the destabilization of the preformed HRG complex (see Fig. 5). (ii) By analogy, ~3-fold higher concentrations of suramin analogues are required to reduce agonist binding to the D_2 dopamine receptor than to increase antagonist binding. This is consistent with competition between activated receptor and suramin analogues for a common site on the G protein α subunit.

A similar competition for interaction with the G protein α

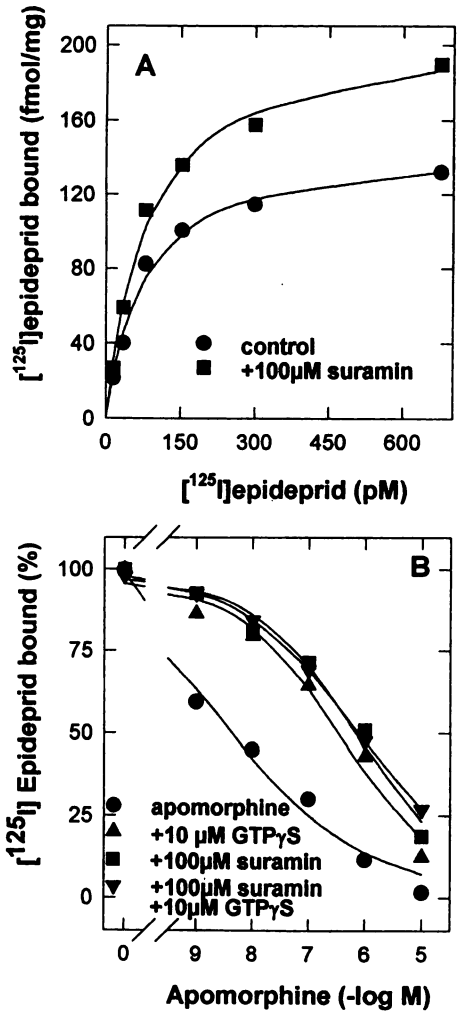


Fig. 7. [^{125}I]Epideprid binding to rat striatal membranes in the absence and presence of suramin. A, Binding was determined in 0.1 ml containing 50 mM Tris·HCl, pH 8, 1 mM EDTA, 5 mM $MgCl_2$, the indicated concentrations of the antagonist [^{125}I]epideprid, and rat striatal membranes (7.5 μg of protein) in the absence (●) and presence (■) of 100 μM suramin for 60 min at 20°. Nonspecific binding determined with 10 μM sulpiride was 5% of total binding in the K_D concentration range. B, The assay conditions were similar to those in A; binding was determined with 100 pM [^{125}I]epideprid, the indicated concentrations of apomorphine (●), apomorphine and 10 μM GTPγS (▲), apomorphine and 100 μM suramin (■), or apomorphine and the combination of 10 μM GTPγS and 100 μM suramin (▲). Specific binding determined in the absence of apomorphine was set at 100%. This value was 1.5 fmol in the presence of GTPγS or suramin and 1 fmol in their absence. Data are mean from duplicate determinations in a single experiment; two additional experiments gave similar results.

subunit can also be observed between suramin and effector (18). The effector binding region of G protein α subunits has been mapped to three noncontiguous regions by site-directed mutagenesis and peptide competition studies as well as with sequence-specific antisera (for a review, see Ref. 29); these form a surface-exposed patch on the protein composed of helix α2 (effector binding site I), helix α3, and the loop connecting α3/β5 (effector binding site II), as well as helix α4 and the loop connecting α4/β6 (effector binding site III) (30–32). The sequences that contribute to the receptor docking site on the α subunit are the carboxyl terminus and the sequence around β6/α5 (for a review, see Ref. 29). The latter is adjacent to and in part overlapping effector binding site III (30–32). The available evidence indicates that the binding site for suramin is in close proximity to this region (18). It is appealing to speculate that binding to this region is sufficient to account for the ability of suramin and its analogues to block receptor/G protein interaction. The possibility that suramin analogues interact with two distinct sites on the G protein α subunit seems unlikely as suramin binds to G protein α subunits with a 1:1 stoichiometry.[1]

Distinct rank orders of potency are observed for the suramin analogues if their ability to block receptor/G protein coupling is compared with their effect on the basal guanine nucleotide exchange rate of defined G protein α subunits. The smaller analogue NF023 decreases the basal rate of GDP release of $G_{i\alpha\text{-}1}$ [$G_{i\alpha}$ is the α subunit of G_i, of which three subtypes exist ($G_{i\alpha\text{-}1}$, $G_{i\alpha\text{-}2}$, $G_{i\alpha\text{-}3}$)] and of $G_{o\alpha}$ (the α subunit of G_o) more potently than suramin (18). These two α subunits are candidate G proteins for coupling to A_1 adenosine and D_2 dopamine receptors in the native membrane (8, 10, 19). In contrast, the large analogues suramin and NF037 are more potent than the smaller NF023 in blocking the interaction between G proteins and A_1 adenosine receptor or D_2 dopamine receptor in the membranes. From these observations, we conclude that NF023 fits better onto $G_{i\alpha}$ as a "plug," preventing the basal release of GDP. However, this does not necessarily mean that NF023 is more potent than suramin in preventing the binding of a receptor to $G_{i\alpha}/G_{o\alpha}$. The surface area covered by suramin and NF037 is larger. This may be more important for uncoupling the receptor. In the presence of NF023, the agonist-activated receptor may presumably find it easier to contact the G protein and to overcome the inhibitory effect than in the presence of suramin.

Two previous studies have investigated the effect of suramin on the interaction between receptors and G proteins. Huang *et al.* (33) tested suramin and several sulfonated aromatic compounds for their ability to reduce high affinity agonist binding to human α_2-adrenergic receptors and reported half-maximal inhibition at 3 μM suramin; this is comparable to the concentration range at which suramin suppresses high affinity agonist binding to the A_1 adenosine receptor. Furthermore, the ability of one compound (L-451,167) to uncouple α_2- and β_2-adrenergic receptors was also compared, and only a modest 2-fold selectivity was seen. In contrast, an earlier report on the effects of suramin in NG 108–15 cell membranes provided strong evidence for selectivity; δ-opioid receptors were uncoupled from pertussis toxin-substrate G proteins, whereas the stimulation of the guanine nucleotide exchange reaction of these G_o/G_i proteins by serum factors was unaffected by suramin (34). Our observations are consistent with the selectivity described in the latter work; high affinity agonist binding to the D_2 dopamine receptor is more sensitive to uncoupling by suramin analogues than that to the A_1 adenosine receptor. The apparent affinity of suramin analogues, however, may be misleading because it depends on the affinity of the receptor for the G protein. The more important finding is the observation that the rank orders of potency are different. Suramin is equipotent with NF037 and 7-fold more potent than NF023 in uncoupling the D_2 dopamine receptor/G protein complex. In contrast, the affinity of suramin for the A_1 adenosine recep-

[1] W. Beindl, T. Mitterauer, M. Hohenegger, A. P. IJzerman, C. Nanoff, and M. Freissmuth, manuscript in preparation.

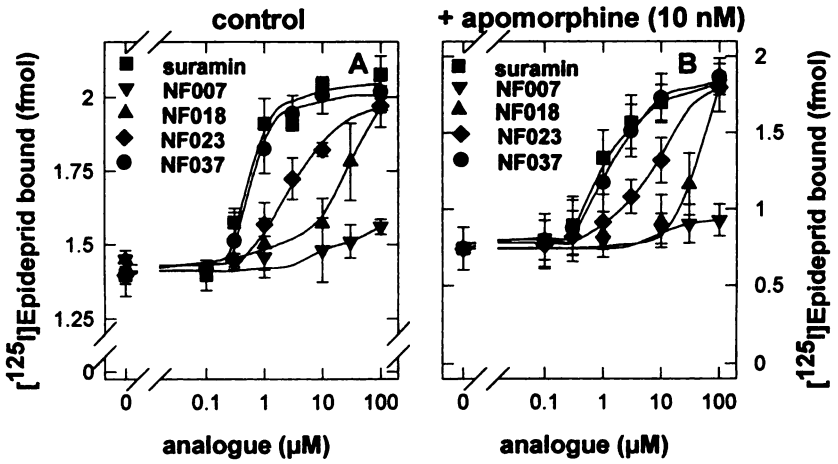


Fig. 8. Effect of suramin analogues on [^{125}I]epideprid binding to rat striatal membranes in the absence (A) and presence (B) of competing apomorphine. The assay conditions were similar to those given in the legend to Fig. 7; the incubation contained 100 pM [^{125}I]epideprid and 10 μg of rat striatal membrane protein and the indicated concentrations of suramin (■) and its analogues (NF007, ▼; NF018, ▲; NF023, ◆; NF037, ●) in the absence (A) and presence (B) of 10 nM apomorphine. Data are mean from three independent experiments carried out in duplicate. *Error bars*, standard deviation.

tor/G protein complex exceeds that of NF037 and NF023 by ~10- and 20-fold, respectively. The potency of the half-molecules is also different; NF007 and NF018 display only low activity on the coupling of A_1 adenosine receptor and G protein. In contrast, NF018 is active on the ternary complex formed by the D_2 dopamine receptor, whereas NF007 barely has an effect.

Reconstitution studies using isolated receptors and defined G proteins indicate distinct coupling preferences for A_1 adenosine and D_2 dopamine receptors; both receptors interact more readily with $G_{i\alpha}$ than with $G_{o\alpha}$, but the $G_{i\alpha}$ subtype selectivity was found to be different (8, 10, 19). Therefore, it is attractive to speculate that the different structure-activity relationship of suramin analogues may arise from the preferential association of distinct receptor/G protein complexes. However, extrapolation of these coupling preferences determined *in vitro* to the situation *in vivo* is limited by the lack of knowledge on the subunit composition of the G protein oligomers with which the A_1 adenosine and D_2 dopamine receptor preferentially associate in the native membrane. Cytoskeletal components participate in higher level organization of receptors and G protein (35); these may contribute to the specificity of receptor/G protein interaction and account for the stringent requirement of a given receptor for a defined G protein oligomer seen in intact cells (3, 15–17). Thus, although the mechanistic interpretation of our findings is incomplete, they nevertheless show that the search for compounds that are highly selective for individual receptor/G protein tandems seems to be feasible.

Suramin has been used for several decades in the treatment of African trypanosomiasis (sleeping disease) and onchocerciasis (river blindness). Renewed interest stems from the finding that suramin has been found to be effective in the treatment of certain types of cancer (36). Suramin blocks the interaction of many growth factors and cytokines with their receptors (37), including platelet-derived growth factor (38), interleukin-2 (39), insulin-like growth factor-1 (40), and type II but not type I interferons (41). G protein-regulated signaling pathways are involved in the control of cellular growth (42), and a pathophysiological role of altered G proteins in several human tumors has been demonstrated (43–45). However, because of its highly polar nature, suramin does not readily cross cell membranes. Therefore, it seems unlikely that the cytostatic effect of suramin, which can be demonstrated on a large variety of cells *in vitro*, is related to its ability to suppress G protein activation. Several tissues, however, accumulate suramin, most notably, the adrenal cortex (46). Treatment with suramin can cause adrenal insufficiency and may be curative in adrenal cancer (46, 47). Suramin depresses not only the growth of adrenocortical cells in culture (46, 48) but also the release of steroid hormones in response to adrenocorticotropic hormone (45, 48, 49). This effect may possibly arise from a block in the interaction between the receptor for adrenocorticotropic hormone and G_s.

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