Biochemical Pharmacology, Vol. 34, No. 4, pp. 491–498, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
Pergamon Press Ltd.

β-ADRENERGIC ACTIVITY AND CONFORMATION OF THE ANTIHYPERTENSIVE SPECIFIC α_2-AGONIST DRUG, GUANABENZ

SOPHIA DIAMANT,* ISRAEL AGRANAT,† AMIRAM GOLDBLUM,‡ SHMUEL COHEN§ and DAPHNE ATLAS*‖

*Department of Biological Chemistry, †Department of Organic Chemistry, ‡Department of Pharmaceutical Chemistry, §Department of Inorganic Chemistry, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel

(*Received* 11 *May* 1984; *accepted* 10 *August* 1984)

Abstract—In recent research a new series of specific drugs, one of which is guanabenz (GBZ, 2,6(dichlorobenzyliden)-aminoguanidine)† has been introduced into the clinical treatment of centrally mediated hypertension. Guanabenz (GBZ) is considered to be among the most specific α_2-adrenergic agonists, acting similarly to clonidine by decreasing the sympathetic outflow from the brain to the peripheral circulatory system. In the present report we show that GBZ displays a significant affinity for β-adrenoceptors. In displacement studies of the iodinated β-antagonist [^{125}I]cyanopindolol (CYP) from turkey erythrocyte membranes, the dissociation constant of GBZ was 3.8 μM. Inhibition of the (-) epinephrine induced adenylate cyclase activity by GBZ is competitive, with an apparent dissociation constant of 30 μM. A similar value was obtained by studies of GBZ's effect on the (-)epinephrine-induced [^{3}H]cAMP accumulation in intact turkey erythrocytes. In view of its unexpected affinity for β-adrenoceptors, we examined the three-dimensional structure of crystalline GBZ. In these studies substantial differences between clonidine and GBZ were observed, despite their strong structural resemblance. These dissimilarities (angle of rotation $\phi = 39.7°$ as compared to 76° in clonidine, and the rotational restriction of clonidine as compared to the greater mobility in rotation of GBZ) could explain the difference of specificity between these two compounds.

The classification of adrenergic receptors into two distinct groups was proposed by Ahlquist [1] in 1948. Further subdivisions of β-adrenoceptors into β_1 and β_2 subtypes [2–4] and of α-adrenoceptors into α_1 and α_2 followed later [5–7]. The classification of α-adrenoceptors into two subclasses, α_1 and α_2, is most accurately based on ligand potency of specific α-adrenergic compounds [8]. The detailed classification of adrenergic receptors has been markedly advanced, following the preparation of highly selective potent agonists and antagonists [9]. Clonidine and guanabenz (GBZ)¶ are considered among the most selective and potent α_2-agonists. These two drugs, resembling α-methyldopa in their antihypertensive action, act centrally; they act as anti-hypertensive drugs by decreasing the sympathetic outflow from the brain to the peripheral circulatory system, as a result of stimulation of central α_2-adrenoceptors (for a detailed review, see refs. 8 and 9). The affinity of both ligands to α_2-adrenoceptors was determined in direct binding studies using [^{3}H]clonidine [10, 11] and a number of specific α_2-antagonists [12, 13]. In the rat *vas deferens*, the specificity of GBZ as an α_2-agonist was comparable to that of xylazine, and was ten times more potent and more selective than clonidine [14, 15]. In other studies, GBZ has been reported to be a potent inhibitor of the pina reflex, which was abolished by yohimbine acting as a specific α_2-antagonist [16]. We have recently demonstrated that GBZ is an antagonist of the (-)epinephrine-induced inhibition of adenylate cyclase activity in human platelets as well as the specific inhibitor of (-)epinephrine-induced platelet aggregation [17].

GBZ resembles both clonidine and the opened-ring clonidine analog, IPRO-4 [2-(2,6-dichlorophenyl)-l-methylguanidine] in (a) its aromatic 2,6-dichlorophenyl ring and (b) in the guanidine moiety, although in GBZ the guanidine moiety is not directly connected to the aromatic ring but through a methyleneimino (–CH=N–) spacer. Previous crystallographic X-ray analysis and quantum mechanical (PCILO) calculations demonstrated the striking resemblance between clonidine and four other related α-adrenergic molecules, supporting a hypothetical model for the interaction between clonidine-like drugs and α-adrenoceptors [18]. In the present report we show that GBZ and clonidine, two potent α_2-agonists with a structural resemblance to each other, differ in their specificity for adrenoceptors. This difference is partly explained by studies of their three-dimensional structures. These differences

‖ To whom all correspondence and reprint requests should be addressed.

¶ Abbreviations used: IBMX, isobutyl-methyl-xanthine; EDTA, ethylenediamine-tetraacetic acid; CYP, cyanopindolol; [^{125}I]CYP, [^{125}I]labeled cyanopindolol; DTT, dithiothreitol; BSA, bovine serum albumin; GppNHp, 5 guanyl-yl- imidodiphosphate; DMEM medium, Dulbecco's modified Eagle's medium; GBZ, guanabenz-(2,6-dichlorobenzyliden)-aminoguanidine; GNA, (napthylmethylene)-hydrazine-carboxamidamide; MNDO, modified neglect of differential overlap; PCILO, perturbative configuration interaction of localized orbitals.

should be taken into account, on the one hand, when using these compounds as antihypertensive drugs and, on the other hand, when studying structural features and properties of the receptor site.

MATERIALS AND METHODS

Materials. [α-^{32}P]ATP (80 Ci/mole) and ^{125}I (~3000 Ci/mmole) were purchased from Radiochemical Centre (Amersham, U.K.); [^{3}H]adenine (5.8 Ci/mmole) was from Zotal (Negav, Israel); [^{3}H]-cAMP was from New England Nuclear (Boston, MA); (-)epinephrine, bovine serum albumin (Fraction V), creatine phosphate, creatine phosphokinase, dithiothreitol, heparin, (±)propranolol, GTP, cyclic AMP, and ATP (synthesized by phosphorylation of adenosine) were from Sigma Chemical Co. (London, U.K.); forskoline was from Calbiochem, and DMEM medium was from Gibo Laboratories (Grand Island, NY). All other chemicals were of reagent grade and quality.

Synthesis and crystallization of GBZ. GBZ was synthesized by condensation of 2,6-dichlorobenzaldehyde (1 eq.) and aminoguanidine (1 eq.) in ethanol. Crystallization of GBZ was from water (yield 60%); m.p. 227° (literature 225–227° [19]).

The synthesis of (2-methylbenzylidene-aminoguanidine-2-tolyl analog) and GNA will be described elsewhere. Benzylidene-aminoguanidine (phenyl-analog) was obtained from Dr. A. Dagan at Makor Chemicals (Israel).

Preparation of turkey erythrocytes. Fresh blood was taken into 200 units of heparin. The cells were washed twice with saline at room temperature, followed by centrifugation at 500 *g* for 10 min, removal of the buffy coat and remains of white blood cells. The washed cells were used for incubation with [^{3}H]-adenine (see below).

Turkey erythrocyte membranes. These membranes were prepared according to a previously described procedure [20].

Determination of cyclic AMP. Cyclic AMP accumulation in intact erythrocytes was measured as previously described [21]. Prior to the addition of ligands, the washed erythrocytes were incubated in DMEM medium containing 2 μCi of [^{3}H]adenine per 10^7 cells. After 2 hr of incubation at 37°, the erythrocytes were washed twice with warm saline, and ligands (dissolved in isotonic solution) were added at zero time. Each reaction system contained ~10^7 cells, 0.5 mM IBMX and ligands, as indicated, in a final volume of 1 ml of 140 mM NaCl and 20 mM Tris–HCl, pH 7.4. The incubation was carried out at 37° and was stopped by boiling during 3 min in a water bath. Carrier solution containing 0.5 mM of each adenine, adenosine, 5-AMP, ADP, cAMP, as well as 5 mM ATP and cAM^{32}P (as internal standard) were added, and [^{3}H]cAMP was isolated by sequential passage of the sample over Dowex and Alumina columns, according to the method of Salomon *et al.* [22].

Preparation of [^{125}I]cyanopindolol (CYP). This radioligand was prepared according to the procedure previously described from Na[^{125}I]carrier-free [23].

Binding assay. Displacement of [^{125}I]CYP in turkey erythrocyte membranes was studied in reaction mixtures containing 50 mM Tris–HCl, pH 7.5, 5 mM $Mg(Ac)_2$ and 2 mM EDTA; 100 pM [^{125}I]CYP, 20–30 μg membrane protein, and the ligand tested at the indicated concentrations. The reaction final volume was 0.5 ml. The assay was carried out for 30 min at 30°. Binding reactions were terminated by diluting the sample with 4 ml of 10 mM Tris–HCl buffer, pH 7.5, followed by immediate filtering of the samples through Whatman GF/C filters. Each filter was then washed by adding 4 ml × 2 of the cold buffer.

Non-specific binding of [^{125}I]CYP was defined as the binding observed in the presence of 1 μM (±)-propranolol. Non-specific binding did not exceed 8–10% of the total binding.

Dissociation constants for the competing ligands were determined according to the method of Cheng and Prusoff [24] using the following equation:

$$K_d = \frac{IC_{50}}{1 + \frac{[I]}{K_I}},$$

where IC_{50} is the concentration of the ligand tested which causes 50% inhibition of [^{125}I]CYP binding, [I] is the [^{125}I]CYP concentration used in the assay, and K_I is the dissociation constant for [^{125}I]CYP measured at saturation, using [^{125}I]CYP.

Adenylate cyclase assay. Adenylate cyclase assays were performed as follows: In a final volume of 0.15 ml containing 50 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 2 mM $MgCl_2$, 5 mM creatine phosphate, 50 units/ml creatine phosphokinase, 0.1 mM cAMP, 1 mM IBMX, 1 μM GTP, 1 mM DTT, 0.2% BSA, 0.1 mM ATP (0.1 mCi/mmole), and 50–100 μg membrane protein, the reaction was carried out at 37° for 20 min. Formation of cAM^{32}P was measured as previously reported [22].

Protein determinations. Membrane proteins were determined according to the method of Lowry [25], using bovine serum albumin as a standard.

X-ray crystal structure analysis. Data were measured on a PW1100/20 Philips four-circle computer-controlled diffractometer. MOK_α ($\lambda = 0.71069$ Å) radiation with a graphite crystal monochromator in the incident beam was used. Intensity data were collected using the ω-20 technique to a maximum of 45°. Intensities were corrected for lorentz and polarization effects. All non-hydrogen atoms were found by using the results of the multan direct method analysis [26]. All crystallographic computing was done on a Cyber 74 computer at The Hebrew University of Jerusalem, using the SHELX 1977 structure determination package.

Quantum mechanical calculations. Quantum mechanical calculations of the basic and protonated forms of clonidine and GBZ were performed by MNDO [27]. Total geometry optimization started with standard bond lengths and angles for each molecule in restricted planar and perpendicular conformations of the aromatic plane vs the five-membered ring (in clonidine) or the imino side-chain (in GBZ). Single molecules were calculated *in vacuo* at 298 K. Relieving the conformational restrictions optimized each 'planar-perpendicular' pair to the same final geometry. This geometry was taken as a

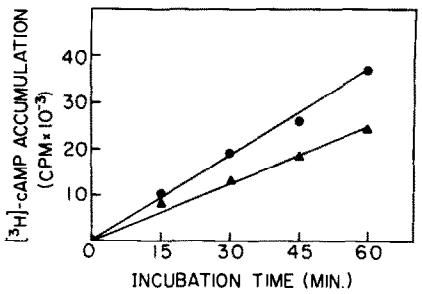

Fig. 1. The inhibitory effect of GBZ on (-)epinephrine-induced accumulation of [^{3}H]cAMP on intact turkey erythrocytes. Incubation of 0.1 ml erythrocyte suspension (~10^7 cells) with 100 μM concentration of GBZ in the presence of 0.5 mM IBMX, in a final volume of 1 ml, was carried out at 37° for 1 hr. The results are expressed in cpm of [^{3}H]cAMP accumulated by 10 μM (-)epinephrine in the absence (●—●) and in the presence (100 μM) of GBZ (▲—▲). In a typical experiment, [^{3}H]cAMP level induced by (-)epinephrine was 38,600 cpm/hr per 10^7 cells.

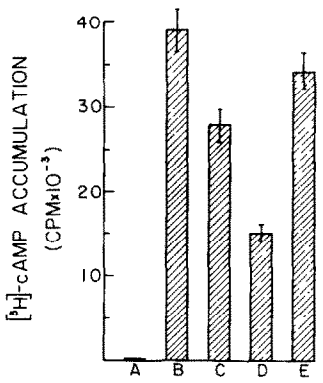

Fig. 2. The effect of guanabenz and other guanidino compounds on the (-)epinephrine-induced [^{3}H]cAMP accumulation in intact erythrocytes. Results are expressed in cpm of [^{3}H]cAMP accumulation/hr per 10^7 cells. (A) In the absence of hormone (basal level); (B) in the presence of 10 μM (-)epinephrine; (C) in the presence of 10 μM (-)epinephrine and 100 μM GBZ; (D) in the presence of 10 μM (-)epinephrine and 100 μM GNA; (E) in the presence of 10 μM (-)epinephrine and 100 μM clonidine. Vertical bars through the data points represent the standard error of the means (N = 3).

basis for a MNDO calculation of rotation about the Cl–N1 (clonidine base and protonated forms) or Cl–C7 bond (GBZ base and protonated forms), with clockwise rotation, starting at the perpendicular conformation. Energies were calculated for rotamers at 10° intervals, all other geometrical parameters being kept constant. Similar rotations were calculated by PCILO for all species [28].

The energy profile of GBZ with its X-ray geometry was compared to that of the quantum mechanical optimized structure, applying MNDO. All calculations were performed on a CDC Cyber 72 at The Hebrew University of Jerusalem computation center.

RESULTS AND DISCUSSION

Intact turkey erythrocytes

GBZ inhibits (-)epinephrine (10^{-5} M) induced [^{3}H]cAMP accumulation in a concentration dependent manner in intact erythrocytes. The IC_{50} (concentration required to reduce the response to 50% of control) is 3.0×10^{-5} M (data not shown). The (-)-epinephrine-induced [^{3}H]cAMP accumulation as a function of time is significantly inhibited ($P < 0.01$) by 100 μM GBZ, as demonstrated in Fig. 1. Similar effects were observed with a naphthalene analog of GBZ (GNA), which at 100 μM inhibited (-)epinephrine-induced [^{3}H]cAMP accumulation by 60%, as compared to 40% obtained with 100 μM GBZ (Fig. 2). Clonidine, however, despite its strong structural resemblance to the above mentioned guanido compounds, at a concentration up to 100 μM did not have any statistically significant influence ($P > 0.5$) on the (-)epinephrine-induced [^{3}H]cAMP accumulation in the intact erythrocyte.

Membranes of turkey erythrocytes

Turkey erythrocyte membranes were exposed to 5, 10 and 100 μM of (-)epinephrine, increasing concentrations of GBZ, in a study of the competitive effects of GBZ and (-)epinephrine. The kinetic data presented in Fig. 3 as a Dixon plot, shows that the most pronounced inhibition of GBZ was observed at 5 μM (-)epinephrine, whereas at 100 μM (-)epinephrine, GBZ (100 μM) had no inhibitory effect. The data shown demonstrate that GBZ acts as a competitive antagonist towards the β-adrenergic agonist (-)epinephrine.

The point of intercept of the slopes in the Dixon plot corresponds to 3.0×10^{-5} M, which is the same IC_{50} value obtained in inhibition of the (-)epinephrine-induced accumulation of [^{3}H]cAMP in intact erythrocytes. GBZ exerts its effects on adenylate cyclase activity only when the enzyme is activated in a reversible manner, namely by hormone stimulation. The irreversible activation of adenylate cyclase by preincubation in the presence of both 10 μM (-)epinephrine and 10 μM GppNHp, is not

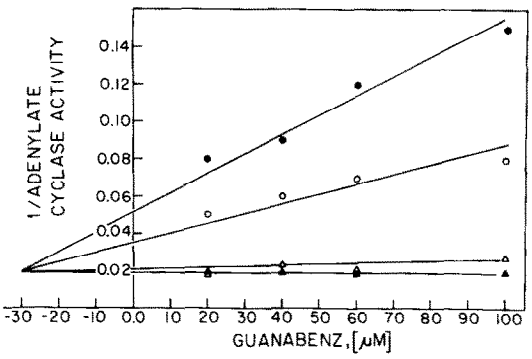

Fig. 3. Kinetic analysis of GBZ-induced inhibition of (-)-epinephrine-induced activation of adenylate cyclase using Dixon plots. (-)Epinephrine-induced adenylate cyclase activation was measured as described in the Experimental Procedures, with 5 μM (-)epinephrine, [●]; 10 μM [○]; 50 μM [△]; and 100 μM (-)epinephrine [▲].

Table 1. Specificity of β-adrenergic action of GBZ in turkey erythrocyte membranes

Agent	Adenylate cyclase activity (pmole/min/mg protein) in absence of guanabenz (100 μM)	in presence of guanabenz (100 μM)
None	1.3 ± 0.02	1.2 ± 0.01
Epinephrine (10 μM)	27.2 ± 0.6	14.6 ± 0.3*
Epinephrine† + GppNHP (10 μM each)	247.2 ± 8.1	255.7 ± 9.0
Forskoline (10 μM)	39.6 ± 2.0	36.6 ± 1.8
NaF (10 mM)	210.0 ± 18.0	304.7 ± 15.7

The results are the mean of three independent experiments (N = 3).

* Statistically significant difference between adenylate cyclase activity in absence and in presence of GBZ.

† Erythrocyte membranes were preincubated with the mixture containing GppNHp and (-)epinephrine prior to the adenylate cyclase assay.

influenced by 100 μM GBZ (Table 1). A similar effect was obtained when using 10 μM (-)isoproterenol instead of (-)epinephrine. GBZ is ineffective in inhibiting either the forskoline or the fluoride activated adenylate cyclase (see Table 1).

Displacement of [^{125}I]CYP in turkey erythrocytes

In displacement of [^{125}I]-CYP from turkey erythrocyte membranes, GBZ acts as an antagonist. It displaces [^{125}I]CYP in a Michaelian manner. A similar displacement pattern was observed with the naphthalene analog GNA (Fig. 4). The K_d values for the two ligands are calculated as described in the Experimental Procedures, and were 2.9×10^{-6} M for GNA and 3.8×10^{-6} M for GBZ, being one order of magnitude lower than the value obtained for antagonism of hormone-stimulated adenylate cyclase activity.

Additional guanidino derivatives such as the 2-tolyl analog and naphazoline, although showing β-antagonistic properties in displacement experiments, are, however, less potent (Fig. 5). The K_d values for these three compounds are 2.7×10^{-5}, 6.0×10^{-5} M and 1.8×10^{-4} M, respectively. Clonidine and UK-14,304, both potent and selective α_2-agonists with a 'closed' guanidino (imidazoline) structure were, nevertheless, practically inactive as displacers of [^{125}I]CYP from its membrane binding site (illustrated in Fig. 5).

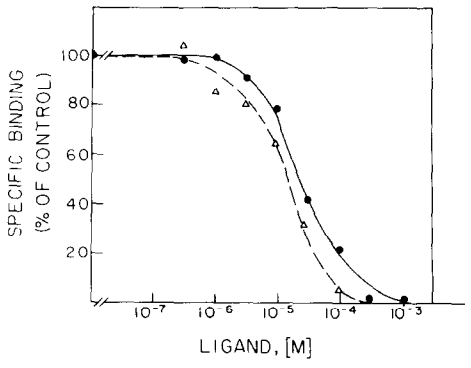

Fig. 4. Binding of [^{125}I]CYP to turkey erythrocyte membranes in the presence of GBZ (●) and GNA (△).

Each incubation mixture contained 20–30 μg membrane protein, and GBZ or GNA at the indicated concentrations. The reaction was started by the addition of 100 μM [^{125}I]-CYP for 30 min at 30°. The corresponding dissociation constants were calculated from the IC_{50} values (see Materials and Methods).

Crystalline GBZ

The crystals are monoclinic, space group $P2_1/C$, with unit cell dimensions: $a = 16.290$ Å; $b = 8.309$ Å; $c = 7.490$ Å; $\beta' = 98.34°$; $Z = 4$; and $R = 0.035$ (Fig. 6). The intramolecular bond distances and angles of GBZ are summarized in Table 1. The stereoscopic ORTEP drawing of GBZ is shown in Fig. 7.

X-ray crystallography and conformation

The aromatic 2,6-dichlorophenyl ring and the methyleneamino-guanidine moiety are each planar within experimental limitations. The X-ray structure of GBZ conspicuously reveals the non-orthogonality of the aromatic ring vs. the guanidine moiety. The angle ϕ between the two planes is only 39.7°. The corresponding angles in clonidine-HCl [29, 30], clonidine-phosphate [31], IPRO-4 [32], and IPRO-4-HCl [32] are 76°, 80°, 81° and 75° respectively. The dihedral angles ψ around the bond connecting the aromatic (2,6-dichlorophenyl)-ring and the remainder of the

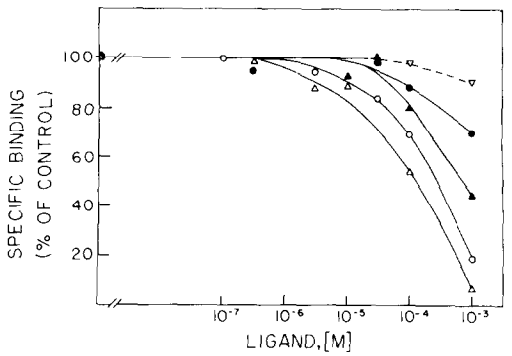

Fig. 5. Binding of [^{125}I]CYP to turkey erythrocyte membranes. Displacement by 2-Me-benzylidene, △; benzilidene-aminoguanidine, ○; naphazoline, ▲; UK-14,304 ■; and clonidine, ▽ (see legend to Fig. 4).

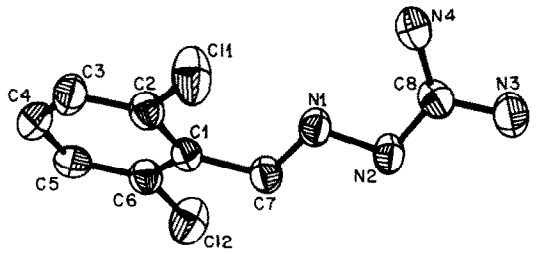

Fig. 6. A view of GBZ, free base. The ellipsoids are scaled to include 50% probability of thermal motion.

molecules are −41.3° (C2-C1-C7-N1) and 139.4° (C6-C1-C7-N1) (GBZ), −77° and 105° (clonidine-HCl), 86° and −92° (clonidine phosphate), −80° and 107° (IPRO-4), and 77° and −107° (IPRO-4-HCl).

The results in GBZ indicate a substantial and significant conjugation through the –CH=N– spacer between the two parts of the molecule.

The two ortho-chlorine atoms vary in their deviation from the plane of the aromatic ring: −0.05 Å (C11) and 0.11 Å (C12). The relevant dihedral angles are −0.89° (C11-C2-C1-C7) and −4.90° (C12-C6-C1-C7). It is more pronounced with C12, due to some overcrowding of C12 and H7. The C12-H7 distance is only 2.72 Å shorter than the sum of the Van-der-Waals radii, 3.0 Å. The short distance of 3.01 Å between C11 and N1 (0.3 Å lower than the sum of the Van-der-Waals radii) should also be noted. It may reflect a certain attraction between these atoms. Their proximity in a planar conformation is probably responsible for the twist of approximately 40° between the aromatic ring and the remainder of the molecule. By contrast, in clonidine and in IPRO-4, the (close to) orthogonal geometry is primarily a consequence of the overcrowding of the chlorines and the (Z)-NH not bonded directly to the aromatic ring.

The previous model [18] was characterized by the following parameters: D, the distance between the cationic center N^+ and the center of the aromatic ring; H, the distance between the cationic center N^+ and the plane of the aromatic ring; and ϕ, the angle between the planes of the aromatic ring and the guanidine moiety. The model called for the following values of these parameters: $D = 4.8 - 5.1$ Å, $H = 0.72 - 1.04$ Å, $\phi = 90° \pm 15°$ (close to 90°). In this model the suggested interactions of clonidine-like drugs at α-adrenoceptors include the following features: (a) an electrostatic attraction between the guanidine function and a negatively charged site of the α-receptors; (b) a hydrophobic interactions between the aromatic nucleus and an electron-deficient area of the α-receptor; and (c) the possible additional formation of a hydrogen bond with the exocyclic NH (not essential). All the clonidine-like drugs included in the model (as free bases and/or protonated species) are characterized by a close to orthogonal conformation of the aromatic ring *vis-à-vis* the guanidine (or imidazoline) moiety. This reflects a very small conjugation, if any, between the two major parts of each drug. As indicated earlier, the crystalline structure of GBZ does not fit this feature of the model.

Table 2. Bond lengths (Å) and angles (degrees) of GBZ

Bond lengths (Å)	Angles (degrees)
C1(1)–C(2) 1.735(2)	C(2)–C(1)–C(6) 115.3(2)
C1(2)–C(6) 1.742(2)	C(2)–C(1)–C(7) 124.4(2)
C(1)–C(2) 1.407(3)	C(6)–C(1)–C(7) 120.3(2)
C(1)–C(6) 1.390(3)	C1(1)–C(2)–C(1) 121.0(2)
C(1)–C(7) 1.475(3)	C1(1)–C(2)–C(3) 117.3(2)
C(2)–C(3) 1.388 (4)	C(1)–C(2)–C(3) 121.7(3)
C(3)–C(4) 1.354(4)	C(2)–C(3)–C(4) 120.4(3)
C(4)–C(5) 1.373(4)	C(4)–C(5)–C(6) 118.8(3)
C(5)–C(6) 1.389(4)	C1(2)–C(6)–C(1) 119.0(2)
C(7)–N(1) 1.275(3)	C1(2)–C(6)–C(5) 117.7(2)
N(1)–N(2) 1.390(3)	C(1)–C(6)–C(5) 123.2(3)
N(2)–N(8) 1.321(3)	C(1)–C(7)–N(1) 120.7(2)
C(8)–N(3) 1.345(3)	C(7)–N(1)–N(2) 115.1(2)
C(8)–N(4) 1.333(4)	N(1)–N(2)–C(8) 109.4(2)
	N(2)–C(8)–N(3) 117.4(2)
	N(2)–C(8)–N(4) 124.3(3)
	N(3)–C(8)–N(4) 118.2(3)

The model parameters of GBZ, IPRO-4 and clonidine salts are summarized in Table 2. It should be noted that the model is somewhat ambiguous with respect to the cationic center in the guanidine moiety. The positive charge may be localized over the whole guanidine skeleton, including the three nitrogen atoms, the hydrogens and the pivotal carbon atom. The 'Y-aromaticity' of the guanidinium and guanidine groups should be considered [33]. None of the atoms in the guanidine moiety of crystalline GBZ fits the D and H values of the model. N2 with $D = 5.11$ Å and $H = 0.55$ Å is the closest one. The structural parameters of crystalline GBZ questioned the validity of the model and called for quantum mechanical calculations of GBZ.

Quantum mechanical calculations

Both GBZ and clonidine, in their basic and protonated forms, are shown by the MNDO total geometry optimization to adopt the orthogonal conformation as their minima. Figure 8 demonstrates the variations in MNDO energies (ΔH°_{298}, kcal/mole) for the relevant rotations of clonidine and GBZ in their basic and protonated forms. The energies reported are differences, $\Lambda(\Delta H^\circ_{298})$, above the molecules' own minimal energies. The energy vari-

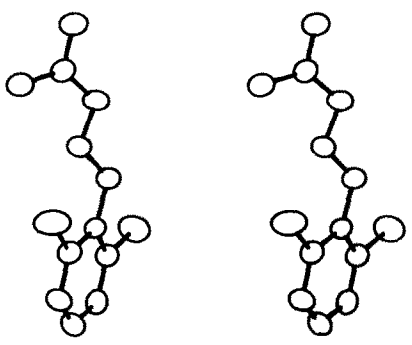

Fig. 7. A stereoscopic view of GBZ.

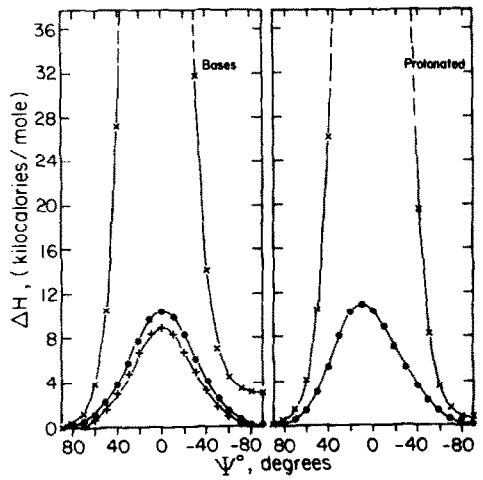

Fig. 8. MNDO calculated energies above minima for rotations about the C1–N1 bond (clonidine, ×—×), the N1–C7 bond (GBZ, ●—●, and X-ray GBZ, +—+). Energies were calculated at 10° intervals for the bases (left) and protonated forms (right). ψ is the dihedral angle at N1 or C7 with respect to the aromatic plane.

ations in clonidine are quite drastic, once the rotation is larger than 30° starting from the minimum (90°). Rotation to the experimental X-ray structure of clonidine ($\psi \sim 75°$) corresponds to a loss of about 1 kcal. GBZ rotations have a similar energetic profile for the computer optimized structure (I) and the X-ray structure (II). Both have low rotation barriers of ~10 kcal. Rotation to the X-ray conformation would require some 3–4 kcal for II and I, respectively.

MNDO energy differences for protonated clonidine are similar to the ones previously reported [18] using PCILO. Other calculations [34, 35] differ widely in their results for energy dependence on

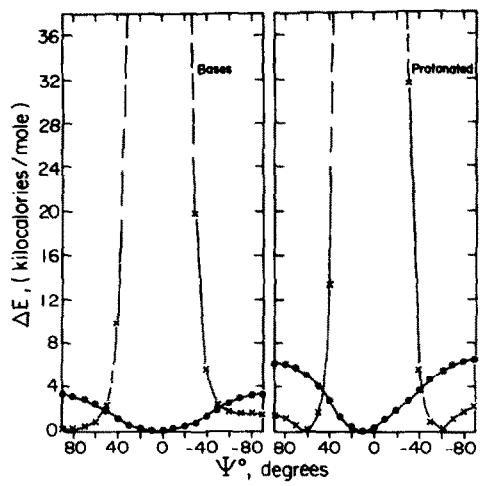

Fig. 9. PCILO calculated energies above minima for rotations about the C1–N1 bond (clonidine, ×—×) and the N1–C7 bond (GBZ, ●—●). Energies were calculated at 10° intervals for the bases (left) and protonated forms (right). ψ is the dihedral angle of N1 or C7 with respect to the aromatic plane.

Table 3. Geometrical characteristics emerging from the X-ray data and quantum mechanical calculations of GBZ and related drugs

	D(Å)					H(Å)						
Guanabenz numbering:		N2	N3	N4	C8		N2	N3	N4	C8	ϕ	ψ
IPRO-4 and clonidine numbering:	N1	N1	N2	N3	C7	N1	N1	N2	N3	C7	(deg)	(deg)
GBZ	3.82	5.11	7.29	5.98	6.02	0.66	0.55	1.34	2.04	1.32	39.7	−41.3	139.4
GBZ H^+ (MNDO)	3.74	5.06	7.31	5.99	6.03	1.09	1.05	2.43	3.24	2.27	77.0	90	90
IPRO-4 [32]		2.82	3.77	4.96	3.68		−0.16	2.15	0.76	−0.89	81.0	−79.0	107.1
IPRO-4·HCl·$\frac{1}{2}H_2O$ [32]		2.82	3.85	5.01	3.77		−0.06	2.16	0.76	0.98	75.1	77.0	−106.7
Clonidine HCl [29]		2.83	3.87	5.00	3.73		−0.02	2.25	1.03	1.05	75.8	−76.5	105.2
Clonidine HCl [30]		2.83	3.87	5.00	3.73		−0.02	2.25	1.03	1.06	75.6	76.2	−105.3
Clonidine phosphate [31]		2.83	3.76	4.98	3.68		0.04	2.40	1.31	1.21	89.5	86.0	−92.3

ϕ—the angle between the planes of the aromatic ring and the guanidine moiety; ψ—the dihedral angles around the bond connecting the aromatic ring and the remainder of the molecule; D—the distance between the cationic center and the center of the aromatic ring; H—the distance between the cationic center and the plane of the aromatic ring.

the angle between phenyl and imidazole rings in clonidine and its analogs. Both find the minima at a nearly perpendicular dihedral angle for the guanidinium carbon, and rotations of an angle larger than 40° from this minimum require large energies in both calculations. PCILO energy variations for the basic and protonated forms of both molecules (Fig. 9) are consistent with the principal results of the MNDO calculations: clonidine in both forms is rotationally restricted relative to the GBZ forms.

MNDO and PCILO are expected to overestimate the energies of the rotamers since geometry optimization was not attempted for each rotamer. Our calculations for geometry optimization in restricted planar and perpendicular conformations for the bases (as compared with the corresponding non-optimized conformation) gave significantly lower enthalpy barriers: ~10 kcal/mole (clonidine) and ~6 kcal/mole (GBZ). Quantum-mechanical results for non-optimized species should thus be treated with caution. The PCILO results predict the planar conformations to be the total minima for GBZ and for protonated GBZ. Clonidine is predicted to adopt a perpendicular conformation, while the protonated form has a minimum at $\psi = 60°$. The latter value differs somewhat from that obtained by the previous PCILO calculation (74°). This difference is probably due to the optimized geometry used in the present PCILO calculation (as compared to the previous X-ray geometry).

PCILO probably overestimates the delocalization energies for the planar structures. Second order delocalization energies are indeed the main source for energy differences among the rotamers in the PCILO calculations. The experimental, X-ray conformations of clonidine and GBZ are at ~1 and ~3 kcal above their minima in the PCILO calculations. The results clearly imply that clonidine in its basic and protonated forms is rotationally restricted as compared to GBZ. Energies of 1–4 kcal may be compensated by electrostatic interactions in the crystal, which may explain the X-ray deviations from the calculated minima. Intermolecular forces in the crystal and the effect of anions may result in a conformational change from the "free" molecules' minima. An exact interpretation of the dihedral angles in clonidine and GBZ would probably require the inclusion of a few molecules, in their crystal geometries, into the calculations.

GBZ and clonidine differ also in another aspect of the previous model [18], namely, charge distribution. Mulliken charges were calculated (MNDO) for the bases and their protonated forms. Upon protonation, the additional positive charge is distributed, in clonidine, over the aromatic nucleus (+0.243), the guanidinium (+0.590) and the methylenes of the five-membered ring (+0.165). In contrast, the additional charge in protonated GBZ resides mostly on the guanidinium moiety (+0.814), while the aromatic part acquires only a small amount (+0.094), similar to the imino 'spacer' (+0.095). The additional positive charge on the guanidine carbon may be singled out: +0.255 in GBZ and +0.083 in clonidine. (Total net charges are +0.600 and +0.621, respectively).

In order to gain further confidence in the MNDO charge distribution, we compared the non-chlorinated analogs of clonidine and GBZ, as basic and protonated forms, in MNDO and the *ab initio* STO-3G basis set [36]. All calculations give a similar distribution of the additional charge in the protonated species. It is interesting to note that protonation of GBZ occurs at N2, although the calculated charge of N2 is lowest among the guanidine nitrogens.

CONCLUSIONS

The results of this study reveal a new aspect of GBZ concerning its structure and specificity. Despite its recognition as a specific α_2-ligand, this compound exhibits a significant affinity for the β-adrenoceptors in turkey erythrocytes, which are known to be devoid of α_2-adrenoceptors. We were able to show that GBZ acts as a specific β-adrenergic antagonist, since (i) it inhibits the (-)epinephrine-induced [^{3}H]cAMP accumulation in intact erythrocytes; (ii) it can displace a specific β-adrenergic antagonist, [^{125}I]CYP, bound to erythrocyte membranes; (iii) it attenuates in a competitive manner the activation of adenylate cyclase by (-)epinephrine; (iv) no effect of GBZ could be demonstrated on irreversibly GppNHp and hormone-preactivated adenylate cyclase activity; (v) GBZ was without effect on adenylate cyclase that was activated independently of the β-adrenergic system, namely by NaF or forskolin.

The characteristics of a β-antagonist are not unique to GBZ because other guanidino compounds also effected binding of [^{125}I]CYP in turkey erythrocytes, the order of potency being: GNA > GBZ > 2-tolyl analog > phenyl analog > naphazoline.

The K_d values obtained for GBZ in binding experiments (3.8×10^{-6}) are one order of magnitude lower than the K_d values determined from adenylate cyclase measurements. This discrepancy between direct binding studies and adenylate cyclase activities was previously described [37], suggesting that components of the adenylate cyclase assay mixture decrease binding ability of ligands to membrane receptors.

The findings presented here were quite unexpected, in view of existing knowledge in the literature. GBZ appears to have most selective pre-synaptic α_2-receptor stimulatory and adrenergic neuron blocking actions [38] (see also ref. 14 for review). None of the reported effects of this drug were ever related to β-adrenergic activity. The ability of GBZ and possibly other guanidino compounds to act as β-receptor antagonists may contribute to their effectiveness as antihypertensive agents for which they are used.

The pK_a value of GBZ is 8.1. Although GBZ exists extensively in the ionized form at physiological pH (7.4, 83.4%), an appreciable and significant percentage exists in the non-ionized form (16.6%). This balance is very similar to that for clonidine (pK_a = 8.05) [39]. The relatively low ratio (5:1) of the ionized:non-ionized form at physiological pH is probably responsible for the facile penetration of the blood-brain barrier. The similarity in the pK_a values of GBZ and clonidine is partially fortuitous. The substantial decrease in the pK_a values in the series guanidine, aminoguanidine and GBZ (13, 11, and

8.1, respectively) may be partially attributed to the inductive and resonance effects of the 2,6-dichlorophenyl group.

The difference in biochemical specificity between clonidine and GBZ could be substantiated by considering the results of the X-ray study and the quantum mechanical studies. GBZ, having a different crystalline conformation than clonidine, also has more rotational freedom which renders it accessible to other conformers and biochemical interactions, in addition to its α_2-specificity. It should be noted that GBZ could not be predicted by the previous model [18] to be a clonidine type drug. More quantum mechanical calculations are under way in an attempt to evaluate the various aspects of the model and its validity.

REFERENCES

1. R. P. Ahlquist, *Am. J. Physiol.* **183**, 586 (1948).
2. K. P. Minneman, L. R. Hegstrand and P. B. Molinoff, *Mol. Pharmac.* **16**, 34 (1979).
3. R. F. Furchgott, *Ann. N.Y. Acad. Sci.* **139**, 553 (1967).
4. A. M. Lands, A. Arnold, J. P. McAuliff, F. P. Ludena and T. G. Brown, *Nature, Lond.* **214**, 597 (1957).
5. S. Z. Langer, *Br. J. Pharmac.* **60**, 481 (1977).
6. J. E. S. Wikberg, *Nature, Lond.* **273**, 164 (1978).
7. C. L. Wood, C. D. Arnett, W. R. Clarke, B. S. Tsai and R. J. Lefkowitz, *Biochem. Pharmac.* **28**, 1277 (1979).
8. R. B. N. W. M. Timmermans and P. A. Van Zwitten, *J. Med. Chem.* **25**, 1389 (1982).
9. O. Schier and A. Marxer, in *Progress in Drug Research*, Vol. 25, p. 9. Birkhäuser Verlag, Basel (1981).
10. D. C. U'Prichard, D. A. Greenberg and S. H. Snyder, *Mol. Pharmac.* **13**, 454 (1977).
11. B. Jarrott, W. J. Louis and R. J. Summers, *Biochem. Pharmac.* **27**, 141 (1979).
12. B. D. Perry and D. C. U'Prichard, *Eur. J. Pharmacol.* **76**, 461 (1981).
13. H. J. Motulsky, S. J. Shattil and P. A. Insel, *Biochem. biophys. Res. Commun.* **97**, 1562 (1980).
14. B. Holmes, R. N. Brogden, R. C. Heel, T. M. Speight and G. S. Avery, *Drugs* **26**, 212 (1983).
15. J. R. Docherty, A. MacDonald and J. C. McGrath, *Br. J. Pharmac.* **67**, 421 (1979).
16. J. Brown and S. L. Handley, *J. Pharm. Pharmac. Commun.* **32**, 436 (1980).
17. D. Atlas, S. Diamant and A. Eldor, in preparation.
18. A. Carpy, J. M. Leger, G. Leclerc, N. Decker, B. Rougot and C. G. Wermuth, *Mol. Pharmac.* **21**, 400 (1982).
19. Shell International Research Maatschappij N.V., British Patent 1,019,120 February 2, 1966, *Chem. Abstr.* **64**, 11132 (1966).
20. A. Levitzki and M. L. Steer, *J. biol. Chem.* **250**, 2080 (1975).
21. D. Schulster, J. Orly, G. Seidel and M. Schramm, *J. biol. Chem.* **253**, 1201 (1978).
22. Y. Salomon, P. Londos and M. Rodbell, *Anal. Biochem.* **58**, 541 (1974).
23. G. Engel, D. Hoyer, R. Berthold and H. Wagner, *Naunyn Schmiederberg's Arch. Pharmacol.* **317**, 277 (1981).
24. Y. C. Cheng and W. Y. Prusoff, *Biochem. Pharmac.* **22**, 3099 (1973).
25. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
26. P. Main, S. E. Hull, L. Lerringer, G. Germain, J. P. Declercq and M. M. Woolfson, *Multan 78. A System of Computer Programs for the Automatic Solution of Crystal Structures from X-ray Diffraction Data.* Universities of York, U.K., and Louvain, Belgium.
27. M. J. S. Dewar and W. Thiel, *J. Am. chem. Soc.* **99**, 4899 (1977).
28. S. Diner, J. P. Malrieu, F. Jordan and M. Gilbert, *Theor. Chim. Acta* **15**, 100 (1969).
29. G. Byre, A. Mostad and C. Rømming, *Acta Chem. Scand.* **B39**, 843 (1976).
30. V. Cody and G. T. DeTitla, *J. Cryst. Mol. Struct.* **9**, 33 (1979).
31. A. Carpy, A. D. Hickel and J. M. Leger, *Cryst. Struct. Commun.* **8**, 433 (1979).
32. A. Carpy, J. M. Leger, C. G. Wermuth and G. Leclerc, *Acta Crystallogr. Sec. B Struct. Crystallogr. Cryst. Chem.* **37**, 885 (1981).
33. J. Klein, *Tetrahedron* **39**, 2733 (1983).
34. C. M. Meerman-van-Benthem, K. van-der-Meer, J. J. C. Mulder, P. B. M. W. M. Timmermans and P. A. Van Zwitten, *Mol. Pharmac.* **11**, 667 (1975).
35. A. D. De Jong and H. van Dam, *J. Med. Chem.* **23**, 889 (1980).
36. W. J. Hehre, R. F. Stewart and J. A. Pople, *J. chem. Phys.* **51**, 2657 (1969).
37. D. Atlas and S. L. Sabol, *Eur. J. Biochem.* **113**, 521 (1981).
38. Y. Miso, K. Fukie and T. Kubo, *Eur. J. Pharmac.* **77**, 177 (1982).
39. R. R. Ruffolo, Jr., E. L. Yaden, J. E. Waddell and J. S. Ward. *Pharmacology* **25**, 187 (1982).