Mechanism of Action of the Diazabicyclononanone-type κ-Agonists

Ulrike Holzgrabe[†] and Wolfgang Brandt[*,‡]

Institut für Pharmazie und Lebensmittelchemie, Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany and Institut für Pflanzenbiochemie, Leibniz-Institut, Weinberg 3, D-06120 Halle, Germany

The 2,4-di-2-pyridyl-3,7-dimethyl-3,7-diazabicyclo[3.3.1]nonan-9-one 1,5-diester **HZ2** was recently found to exhibit high affinity and selectivity to the κ-opioid receptor (KOR) in combination with an unusually long duration of action. Docking of **HZ2** to the putative binding site model of the KOR revealed **HZ2** to be tightly sitting in the binding pocket. Strong interactions, especially salts bridges between the protonated nitrogens of **HZ2** and the glutamic acids 209 and 297, nicely explain the high affinity of **HZ2** to the KOR. A formation of a hemiaminal bond between the keto carbonyl group of **HZ2** and a lysine residue (Lys200) may explain the long duration of action.

Introduction

The pharmacological effects of opioid-type drugs are mediated by the three major subtypes of the G-protein-coupled receptors, μ-, κ-, and δ, recently renamed MOR, KOR, and DOR.[1] Whereas drugs binding to the MOR are characterized on one hand by a very high analgesic potency and on the other hand by a high incidence of side effects, such as respiratory depression, constipation, tolerance, myosis, and feelings of euphoria, agonists of the KOR produce analgesia without the undesirable effects of the μ-opioids, e.g., the respiratory depression and severe inhibition of gastroinstestinal transit. However, the stimulation of the KOR is associated with side effects, such as sedation and diuresis.[2]

The KOR is an important focus of efforts identifying drugs for treatment of strong pain caused by surgery or cancer. Ketocyclazocin, the first recognized nonpeptide KOR-agonist derived from the benzomorphane group, exhibited only a slight preference for the KOR.[3] At the beginning of the 1970s, arylacetamide compounds of high affinity to the KOR, combined with a low affinity to the other opioid receptor subtypes, were found. As expected, for example, U-50,488, one of the first arylacetamides, did not produce respiratory depression, constipation, and tolerance.[4] However, the first compounds of this type in clinical trials for postsurgical pain, spiradoline and enadoline, have been anbandoned due to dose-limiting dysphoria.[5] To avoid the side effects associated with the CNS, peripherally acting KOR-agonists are at present the focus of interest for use in inflammatory hyperalgesia. One such example is asimadoline, which has utility in treating rheumatoid arthritis.

Recently, **HZ2**, the 3,7-dimethyl-2,4-di-2-pyridyl-3,7-diazabicyclo[3.3.1]nonan-9-one 1,5-diester, was found to exhibit high affinity and selectivity to the κ-opioid receptor.[6] The radioligands for MOR, DOR, and KOR sites used were [3H]naloxone, [3H]Cl-DPDPE, and [3H]-CI977, respectively. K_i values at opioid receptors, KOR,

MOR, and DOR in membrane preparations of the rat brain were found to amount to 0.015 ± 0.004 μM (KOR), >1 μM (MOR), and >10 μM (DOR).[7] In addition, in acute antinociceptive tests using thermal, chemical, and electrical stimuli in rats, mice, and rabbits, **HZ2** showed a unusual long duration of action (>7 h) after both intravenous and oral administration. Effects of comparable length in time were also observed in isolated organ tests, e.g., in the twitch test performed in guinea pig ileum or rabbit *vas deferens*.[7] Furthermore, in some cases the washing out of **HZ2** turned to be difficult. The in vivo and in vitro results together indicate that the prolonged activity is likely due to a strong interaction with the receptor protein rather than due to the pharmacokinetics.

Since **HZ2** does obviously not resemble the aforementioned κ-agonists, structure(conformation)−activity relationships (SAR) were studied in order to find the active conformation. Ketocyclazocine (**KCZ**), the arylacetamide compounds U50,488, U69,593, CI977, EMD61,-753, and some analogues, were compared with **HZ2** with respect to their conformational behavior, molecular electrostatic potential, molecular hydrophobic potential, and hydrogen bonding potential. The analysis of the so-obtained SAR revealed a chair-boat conformation of a protonated **HZ2** characterized by an almost parallel orientation of the C9 carbonyl group and the $^+$N7−H group (protonated) in conjunction with at least one aromatic ring to be the pharmacophoric arrangement.[8] Since this model derived from an int*ra*molecular SAR analysis does not consider the receptor surrounding, it is not able to explain the long duration of action of **HZ2** and the SAR obtained from systematical variations of the substituents with respect to the substitution pattern of the aryl rings, and the substituents attached to the nitrogens in position 3 and 7: Whereas the pyridine rings in 2 and 4 position can be replaced with *p*-methoxy-, *m*-hydroxy-, and *m*-fluoro-substituted phenyl rings without a loss of affinity to the κ-receptor,[9] the nitrogen N3 can be substituted with a hydrogen or a methyl group only.[10] Increasing the size of the substituent at this position resulted in a complete loss of affinity, which could be explained by the change of the stereo-

* Corresponding author. Phone: ++49-345-55821360. Fax: ++49−345−55821309. E-mail: wbrandt@ipb-halle.de.
† Universität Würzburg.
‡ Leibniz-Institut.

chemical arrangement: The increase of the bulk of this substituent produces a boat/chair conformation in conjunction with a trans configuration of the pyridine rings, which results in a structure not fulfilling the aforementioned pharmacophore model.[11] Similar pharmacological observations were made with increasing the bulk of the N7 substituent.[12]

To explain the SAR found and in order to predict compounds of higher affinity, the compounds should be docked to the KOR protein. For example, Ronsisvalle and co-workers[13] were able to build a pharmacophore model considering arylacetamide compounds and benzomorphane derivatives by using the model of the KOR developed by Portoghese and co-workers in 1996.[14] Similar studies have been carried out by Subramanian and co-workers and Iadanza and co-workers.[15,16] These authors considered the binding inside the helix bundle, where the protonated nitrogen of the bound agonist forms a salt bridge to Asp138 carboxylate. Another model of KOR has been developed by Wan and co-workers, who included modeling of the three extracellular loops.[17] They could show that dynorphin A may interact with Glu209 of the second extracellular loop, which demonstrates that both binding inside the helix bundle as well as at the extracellular loops are of importance for the binding of the ligands. However, diazabicyclononanones have not yet been considered for docking studies to KOR, and a second potential binding site at the extracellular loops as indicated by site-directed mutagenesis studies for the DOR[20] has also not yet been considered for KOR.

The recently published high-resolution X-ray structure of bovine rhodopsin,[18,19] which prompted the revision of most of the G-protein-coupled receptor models, opens the principal opportunity to also model the conformations of the extracellular loops. However, this X-ray structure represents a conformation in an inactive state of the G-protein-coupled receptor, whereas the diazabicyclononanones are agonists and may cause changes in the conformation of the KOR. Recently a model of the active state conformation of bovine rhodopsin based on NMR data has been published which would in principle allow to take this model as template for homology modeling of KOR.[20] However, this model contains a lot of uncertainties such as many close contacts, and torsional angles outside allowed regions of a ramachandran plot, and probably most important the docked retinal collapses completely with the side chain of Trp265. All these imponderabilities of this NMR-model prompted us to use the X-ray structure of bovine rhodopsin as a template for our homology modeling of KOR.

Thus, the aim of this study was to build a new KOR model and to dock diazabicyclononanones, KCZ, and arylacetamides into the receptor protein. The pattern of interactions between the KOR and the diazabicyclononanones should on one hand explain the affinity of the ligands to the receptor and on the other hand the long duration of action.

Results and Discussion

Reactivity of the Diazabicyclo[3.3.1]nonanone 1,5-Diester Skeleton. In a previous study the formation of an intramolecular hemiaminal of **HZ2** was observed in acidic media.[21] The reaction pathway was hypothesized to start off with the protonation of the carbonyl oxygen (at C9). In contrast to the observation of a Retro-Mannich reaction, which has already been extensively investigated in connection with the mechanism of cis−trans isomerization,[22] the positive charged carbonyl group formed a covalent bond to the nitrogen of a pyridine ring in the axial position. To gain more insight in the hemiaminal formation semiempirical PM3 and ab initio DFT (B3LYP(6-311G*++) calculations were carried out herein. We calculated energies (DFT) and the heats of formation (PM3), respectively, of possible states of single protonation of **HZ2** (N3, N7, and keto) and the transition state for the formation of the hemiaminal. From the thermodynamic point of view, the protonation at N3 (DFT= −933421.4 kcal/mol, PM3: ΔH_f = 38.9 kcal/mol) is most favored. In comparison to this value the protonation at the keto carbonyl oxygen (DFT = −933362.8 kcal/mol ΔH_f = 64.8 kcal/mol) is less favorable. However, upon building up the hemiaminal with the protonated oxygen the energy (−933419.0) and the heat of formation drops down to ΔH_f = 45.2 kcal/mol. This is on one hand quite close to the heat of formation of the N3 protonated **HZ2** and demonstrates that the energy gain for the formation of the hemiaminal is 56.2 kcal/mol (19.6 kcal/mol) in the case the oxygen atom is protonated, and a trans-configuration is adopted. These results clearly indicate that the most crucial step in the hemiaminal formation is the first step, the protonation at the keto-carbonyl oxygen. This may happen either by a temporarily or accidental jump from the N3 or N7 protonated nitrogen atoms, which are in close vicinity to the oxygen or by a proton that is delivered by an external proton donor, which will be discussed below.

In addition, we investigated the activation barrier (kinetics) for the formation of the hemiaminal by means of reaction coordinate calculations. The result of this calculation (PM3) is graphically represented in Figure 1. Transition state search with ab initio DFT gave no result, but the final structure with the formed hemiaminal was obtained. A very small activation barrier was obtained by the PM3 calculations (PM3 = 1.2 kcal/mol), both indicating that there is indeed no real kinetic hindrance to form the hemiaminal.

The most striking result of the calculation is the fact that the formation of the hemiaminal bond occurs as soon as the carbonyl group is protonated. Thus, it can be imagined that in the cases where receptor pocket protons are present, corresponding aminal, acetal, or thioacetal linkages can be formed with lysine, tyrosine and serine, and cysteine residues, respectively. To estimate the thermodynamic probability of the reaction with each amino acid, the heats of formation of the **HZ2**, the *N*-acetyl and *N*-methylamide amino acid residues (we used the N-terminal and C-terminal protected residues to mimic adjacent residues in the receptor) as well as the corresponding products were calculated by means of the semiempirical PM3 and ab initio DFT (B3LYP(6-311G*++) calculations. The so-obtained reaction enthalpies ΔH_r were found to show small positive values for both the ab initio and PM3 calculations for tyrosine (see Table 1). In the case of cysteine, the ab initio DFT calculations highly favor a reaction, whereas
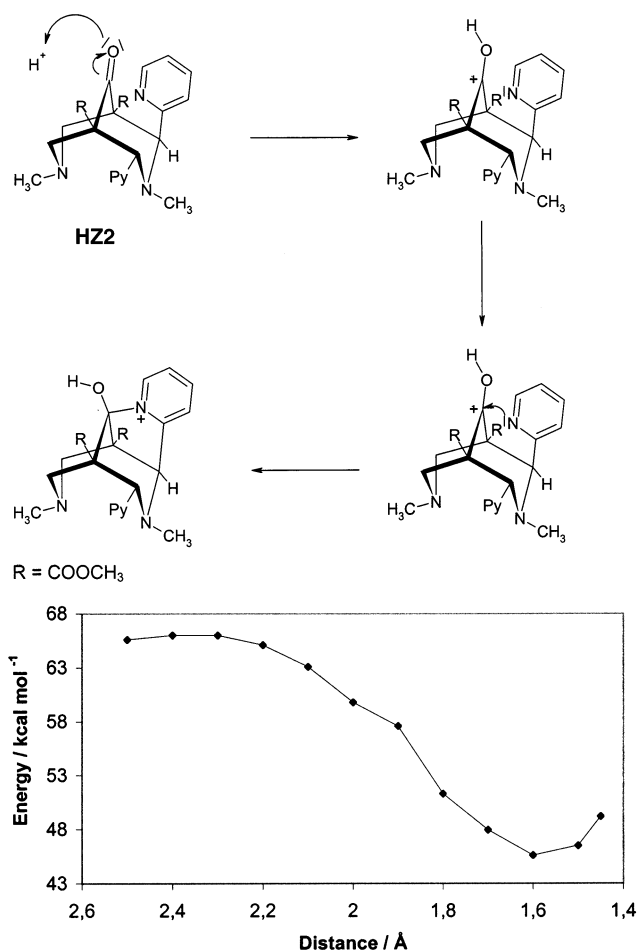
Figure 1. (a) Reaction pathway of the formation of the hemiaminal. (b) Schematic representation of the energy change for the formation of the hemiaminal bond by shortening of the distance between the nitrogen of the pyridine ring and the carbon atom C9 based on semiempirical PM3 calculations.

Table 1. Resulting Reaction Enthalpies (in kcal/mol) Based on ab Inito B3LYP (6-311G*+) and PM3 (in brackets) Calculations of HZ2 with Amino Acid Residues

HZ2(trans)	Ser		HZ2-Ser	$\Delta_r H$
−933166.3	−358316.5	→	−1291482.3	0.52
(−101.1)	(−133.1)		(−225.1)	(9.1)
HZ2(cis)				
−933166.0	−358316.5		−1291487.0	−4.52
(−98.2)	(−133.1)		(−230.1)	(1.2)
HZ2(trans)	Cys	→	HZ2-Cys	
−933166.3	−560921.7		−1494156.1	−68.03
(−101.1)	(−76.7)		(−164.3)	(13.5)
HZ2(cis)				
−933166.0	−560921.7		−1494163.0	−75,24
(−98.2)	(−76.7)		(−164.4)	(10.5)
HZ2(trans)	Tyr	→	HZ2-Tyr	
−933166.3	−503321.1		−1436465.8	21.59
(−101.1)	(−110.6)		(−197.3)	(14.4)
HZ2(cis)				
−933166.0	−503321.1		−1436466.7	20,42
(−98.2)	(−110.6)		(−197.1)	(11.7)
HZ2(trans)	Lys	→	HZ2-Lys	
−933166.3	−420101.1		−1353276.7	−9.30
(−101.1)	(52.9)		(−68.9)	(−20.7)
HZ2(cis)				
−933166.0	−420101.1		−1353277.1	−10.00
(−98.2)	(52.9)		(−58.1)	(−12.8)

from the PM3 calculations, positive reaction enthalpies were observed. The latter might be caused by insufficient parametrization of the sulfur atom in PM3.

However, in the case of lysine both methods give negative values clearly indicating that this reaction is a likely reaction in this series. Thus, such a reaction has to be taken into consideration when docking the molecules into the receptor protein.

Docking HZ2 into the Receptor. Most models of GPCRs consider a binding site of the ligands inside the seven transmembrane helix bundle.[15,16,23] There is a highly conserved aspartic acid residue which may represent the anionic binding site for all ligands possessing a positively charged nitrogen atom. However, site-directed mutagenesis studies[24] demonstrated for the DOR that another binding site occurs very likely between the second and third extracellular loops. Recently Zhang et al. investigated the putative conformation of the second extracellular loop of KOR by means of NMR based on a synthetic peptide of this loop in dodecylphosphocholine micelle environment and propose a model of KOR.[25] These authors describe that dynorphin A interacts with residues within loop 2 at the sequence Val201 to Cys210.

Another study which describes modeling investigations of a model of KOR together with dynorphin A(1−8) showed similar results.[17] These authors detected interactions of dynorphin not only with several amino acid residues of the transmembrane helices but also with residues of all three extracellular loops in particular with extracellular loop two. For example, Arg6 of dynorphin is predicted to interact with Glu209 but also with Arg202, Val207, and all residues between Cys210 and Gln213. This clearly indicates the importance of the extracellular loops for binding of κ-selective ligands.

Our molecular modeling and docking studies strongly support the existence of both binding sites for the KOR. As a test of our hypothesis, we docked nor-BNI to the model of KOR. The docking studies clearly show that one part of nor-BNI is able to interact with one binding site inside the seven transmembrane helix bundle and the other part with its protonated nitrogen at the site at the extracellular loops (Figure 2a). The protonated nitrogen atom which is located inside the helices forms a salt bridge to Asp138 (Figure 2b) An additional strong hydrogen bond is formed between Glu209 and the hydroxyl group of nor-BNI. Hydrophobic interactions with Leu295 and Ile316 further stabilize the interactions with the receptor. If the ligand is docked in this way to the receptor (which is commonly accepted), the other half mirror part of the ligand will never be able to reach a postulated dimer of the receptor. However, as displayed in Figure 2b, the other protonated nitrogen atom forms a strong salt bridge to Glu297 which is part of the extracellular loops. Further studies with other ligands which highly support this model will be published elsewhere.

Moreover, interactions of dynorphin A with Glu209, Ile316, and Glu297 of KOR have also been observed by Wan et al.[17] These results are highly supported by chimeric receptor research showing the middle domain of the fourth transmembrane helix and the second extracellular loop to be critical for high affinity binding of prodynorphin peptides to KOR.[26]

Docking studies of **HZ2** clearly showed a preferred binding at the extracellular loops as shown in Figures 3 and 4. The docking revealed some very interesting
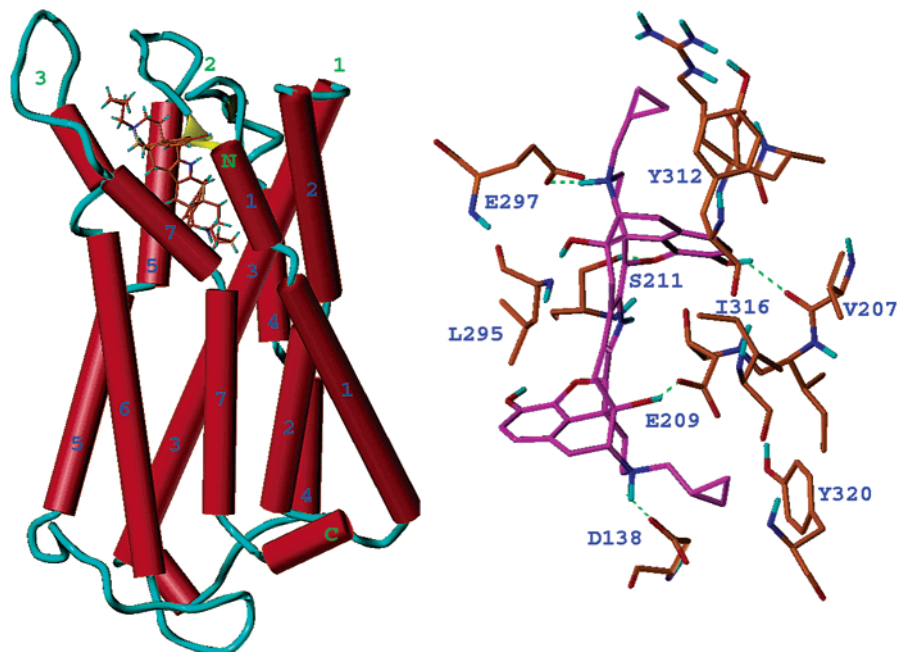
Figure 2. (a) Overview of the docking arrangement of nor-BNI to the KOR. (b) Most important interactions sites of nor-BNI with both ligand binding sites of KOR.
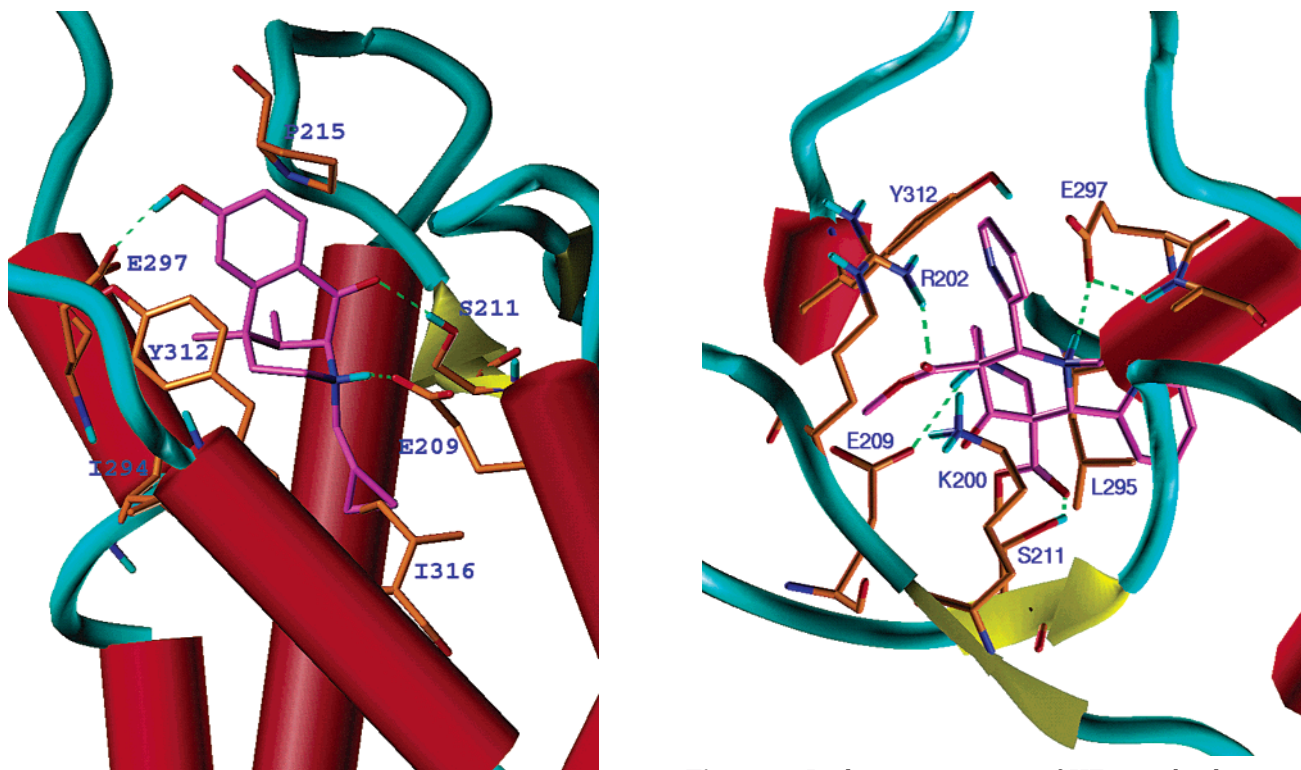

Figure 3. Docking arrangement of ketocyclazocin to a binding site of KOR between the extracellular loops two and three. Red cylinders represent the transmembrane helices; the light greenish-blue tubes are the extracellular loops. Hydrogen atoms linked to a carbon atom are not shown for better overview.

Figure 4. Docking arrangement of **HZ2** at a binding site of KOR between the extracellular loops two and three. Most important is the formation of two salt bridges with E209 and E297. The side chain of K200 is close to the keto carbonyl group of **HZ2** which may allow a proton jump to the keto carbonyl oxygen atom.

characteristic interactions. Both compounds form a salt bridge to Glu209 in addition to a hydrogen bond of the carbonyl oxygen atom to Ser211. Ser211 has also been shown to be important for interaction with Arg7 of dynorhin A in the aforementioned paper of Wan et al.[17] In comparison to the docking arrangement of nor-BNI only the side chain of Glu209 is slightly rotated.

Furthermore, the phenolic hydroxyl group of KCZ builds a hydrogen bond to Glu297. This mode of interactions is in agreement with our model of SARs of κ-selective opioids previously published.[8] A nearly parallel orientation between the NH-group of the protonated nitrogen atom and a carbonyl group was proposed as one of the most important features of κ-selective compounds. In
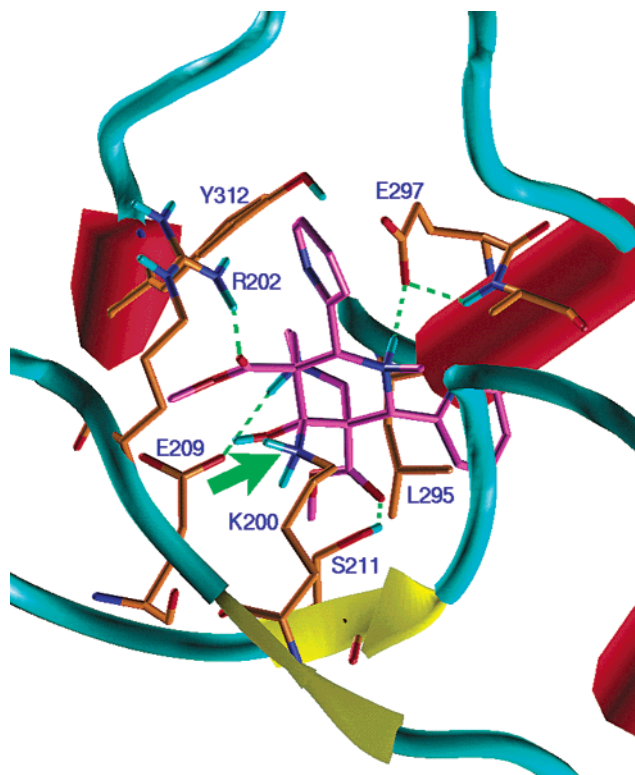
Figure 5. A covalent bond is formed between the side chain of K200 and the ketocarbonyl carbon atom of **HZ2** without any major conformational changes or hindrance.

Figure 6. Docking arrangement the *m*-fluorophenyl-substituted diazabicyclononanone at a binding site of KOR between the extracellular loops two and three. The fluoro atoms form hydrogen bonds to Y312 and K227. Representation of the helices and loops was omitted for better clearness.

Figure 7. Docking arrangement the *p*-methoxyphenyl-substituted diazabicyclononanone at a binding site of KOR between the extracellular loops two and three. The methoxy groups form hydrogen bonds with the hydroxyl functions of T302 and K227. Representation of the helices and loops was omitted for better clearness.

the case of KCZ, hydrophobic binding areas are additionally formed with Ile316, Pro215, and the phenyl ring of Tyr312, whereas one pyridine group of **HZ2** exhibits hydrophobic interactions with Leu295 and Tyr312.

HZ2 is characterized by two nitrogens in position 3 and 7. Due to the pK_a values of 8.1 and 10.9, both nitrogens are protonated at the pH value of 7.4, which is typical for a physiological medium. Along with a double protonation, **HZ2** changes conformation from a chair/chair to a chair/boat arrangement. Thus, in the case where the second nitrogen atom is protonated, an additional salt bridge may be formed. The docking revealed a second salt bridge between the protonated N7-nitrogen atom of **HZ2** and to Glu297 to be true. The formation of two salt bridges between the bicyclononanones and the KOR is a feature which is not found with any other ligand and may be a reason for the high affinity to the KOR. The fact that corresponding 3-oxa-7-azabicyclononanones, which can be monoprotonated (at N7) only, do not show substantial affinity to the KOR[8] strongly supports the importance of the two aforementioned salt bridges.

Furthermore, it was found that the pyridine rings in 2 and 4 position can be replaced with *p*-methoxy-, *m*-hydroxy-, and *m*-fluoro-substituted phenyl rings without a loss of affinity to the κ-receptor.[9] We docked all these **HZ2** analogues to the extracellular binding site. Representatively, the docking arrangement of the compounds with the *m*-fluoro- and *p*-methoxy-substituted phenyl rings are shown in Figures 5 and 6.

Beside the interactions of the ligands with KOR discussed above, both *m*-fluoro atoms can form hydrogen bonds, one to the phenolic hydroxyl group of the side
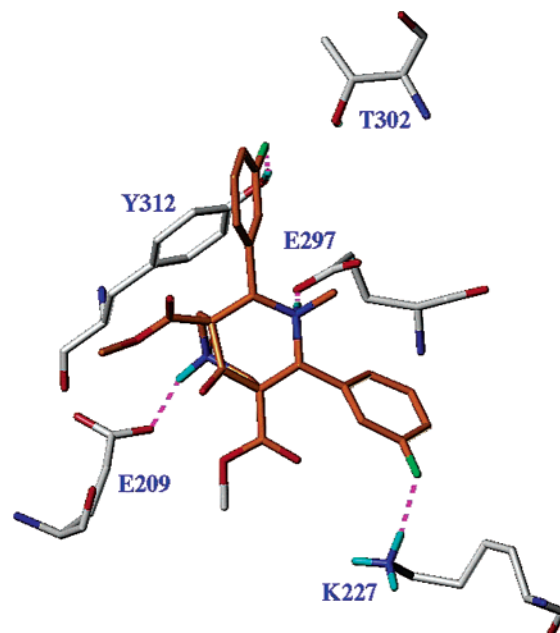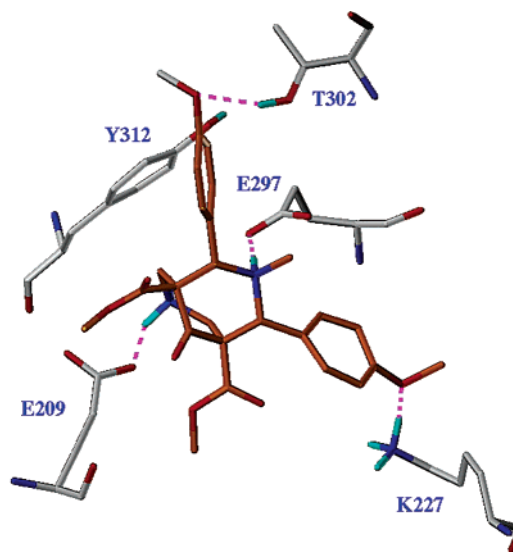
chain of Tyr312 and the other to Lys227. These types of interactions are also found for the *m*-hydroxy-substituted phenyl rings. In the case of the *p*-methoxy-phenyl-substituted compound the attractive interactions are slightly different for one position. Instead of tyrosine the hydroxyl group of Thr302 functions as a hydrogen bond donor for the methoxy group of the diazabicy-clononanone (Figure 7). These interactions may explain the retained affinity of all three derivatives of **HZ2** but also the reduced activity in the case of nonsubstituted phenyl compounds, because these are not able to form any hydrogen bond.

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           1                                                                                  80
1F88A   WQFSMLAAYM FLLIMLGFPI NFLTLYVTVQ HKKLRTPLNY ILLNLAVAD- LFMVFGGFTT TLYTSLHGYF VFGPTGCNLE
KAPPA   AIPVIITAVY SVVFVVGLVG NSLVMFVIIR YTKMKTATNI YIFNLALADA LVTTTMPFQS TVY--LMNSW PFGDVLCKIV

           81                                                                                 160
1F88A   GFFATLGGEI ALWSLVVLAI ERYVVVCKPM S--NFRFGEN HAIMGVAFTW VMALACAAPP LVGWSRYIPE GMQCSCGIDY
KAPPA   ISIDYYNMFT SIFTLTMMSV DRYIAVCHPV KALDFRTPLK AKIINICIWL LSSSVGISAI VLGGTKVRED VDVIECSLQF

          161                                                                                 240
1F88A   YTPHEETNNE SFVIYMFVVH FIIPLIVIFF CYGQLVFTVK EAAAQQQESA TTQKAEKEVT RMVIIMVIAF LICWLPYAGV
KAPPA   PDDDYSWWDL FMKICVFIFA FVIPVLIIIV CYTLMILRLK SVRLLSG-SR EKDRNLRRIT RLVLVVVAVF VVCWTPIHIF

          241                                                             289
1F88A   AFYIFTHQGS DFGPIFMTIP AFFAK--TSA VYNPVIYIMM NKQFRNCM
KAPPA   ILVEALGSTS HSTAALSSYY FCIALGYTNS SLNPILYAFL DENFKRCFR
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Figure 8. Alignment of the sequences of bovine rhodopsin and of the κ-opioid receptor (N-terminal and C-terminal residues were cut). Underlined amino acid residues indicate α-helical transmembrane domains.

Further inspection of the binding site reveals a lysine residue (Lys200) in close vicinity to the keto carbonyl group C9 of **HZ2**. The experimentally found hemiaminal formation and corresponding semiempirical calculations, showing preferred reaction with a lysine, make a reaction between Lys200 and **HZ2** to be likely. The proton from the protonated side chain nitrogen of Lys200 may jump in an initial step to the keto carbonyl oxygen. As demonstrated above, the formation of a covalent bond between the side chain of Lys200 and **HZ2** is subsequently possible. In Figure 4 the force field optimized arrangement of such a covalent complex is shown. There is no spatial hindrance or any considerable conformational changes necessary to form the covalent bond. Thus, we suggest, that the formation of two salt bridges and of the covalent hemiaminal bond may explain the high affinity of **HZ2** to the KOR as well as the long duration of action. However, the formation of the hemiaminal is a reversible procedure.[27] Thus, **HZ2** binds to the receptor only for a limited amount of time, resulting in an appropriate duration of action.

Conclusion

Taken together, **HZ2** is tightly fixed in the binding pocket with a couple of strong interactions, e.g., two salt bridges. From close inspection of the binding pocket it can be easily understood that the N3-substituted compounds which have adopted a boat/chair conformation and trans-configuration of the pyridine rings did not show any affinity to the KOR. Compounds of such a stereochemical arrangement cannot fit into the binding pocket. Additionally, there is no space for bulky alkyl substituents attached to N7. The high affinity of bicyclononanones with *m*-hydroxy, *m*-fluoro-, and *p*-methoxy-substituted phenyl rings can also be explained based on the docking studies.

Interestingly, in this docking model the amino group of lysine 200 is found to be in close vicinity to the keto carbonyl group C9 of **HZ2**. Thus, the proton transfer from the lysine-NH_3^+ group of the receptor to the C=O of **HZ2** is likely to occur, and the formation of the above-mentioned lysine-**HZ2** hemiaminal seems to be inevitable.

Finally, it is worth mentioning, that the pharmacophore model previously derived from the analysis of int*r*amolecular SAR considering the ligands was only confirmed by the docking study presented here.

Experimental Section

Molecular Modeling. The model of the κ-opioid receptor was developed by homology modeling using bovine rhodopsin[18,19] as a template and the COMPOSER[28,29] module of the SYBYL molecular modeling package.[30] For the alignment of the sequence of bovine rhodopsin and those of the κ-opioid receptor, the N-terminal residues (34 of rhodopsin and 56 of the κ-receptor) as well as of the C-terminus (9 of rhodopsin and 38 of the κ-receptor) were removed. The resulting alignment using the BLOSUM62 matrix is displayed in Figure 8. Underlined residues indicate the α-helical domains of the experimental structure of rhodopsin and the predicted transmembrane domains of the receptor.[31] The alignment clearly shows the coincidence of all helical regions with the experimental structure of rhodopsin.

Whereas the seven transmembrane helices showed considerable homology to bovine rhodopsin, it was not simply possible to form the loop conformations. The model of the seven transmembrane helices resulting from COMPOSER was minimized by, first, fixing the backbone dihedral angles and optimizing the side chains only. Afterward the side chains were fixed, and the backbone angles were optimized using in both cases the AMBER 4.0 force field.[30] The stereochemical quality of the structure was examined with PROCHECK[32] which showed all residues inside all criteria, such as percentage of residues in most favored and favored regions, ω angle standard deviation, H-bond energy, and others. Extra- and intracellular loops were investigated separately. A first crude model of the loops was also created by means of COMPOSER based on alignment with bovine rhodopsin, and loop search algorithms included in SYBYL. On the basis of the alignment, the disulfide bond between the cysteine residues occurring in the third helix and the second extracellular loop could be closed without difficulty. Simulated annealing runs, heating the compound to 700 K for 2000 ps and cooling afterward to 100 K within 1000 K were performed 30 times. The AMBER 4.0 force field was applied. The low temperature conformations were minimized to an energy change of 0.01 kcal/mol. The energies of the resulting conformations were checked, and for analysis of the stereochemical correctness in accordance with native amino acid conformation, PROCHECK was applied. The low energy conformations with no residue in disallowed areas of the Ramachandran plot were then used for docking studies of several κ−selective compounds. For this purpose the docking program GOLD[33,34] was used. The standard default settings were used. For each ligand, 30 docking arrangements were allowed to be created by GOLD. Binding sites were found close to the Asp138 inside the helix bundle but also at the extracellular loops two and three. The latter is discussed here only.

Quantum chemical calculations were performed with Gaussian98[35] and MOPAC (PM3) implemented in SYBYL. All compounds were first optimized using PM3 and subsequently refined by ab initio DFT B3LYP(6-311G^{*+}).

pK_a Values. The pK_a values were measured potentiometrically in water using the Sirius PCA101 apparatus (Heath

Scientific, UK). Exactly 0.002−0.005 g of the substances was dissolved in 8−12 mL of aqueous dioxane (50%) and diluted to 20.0 mL with a 0.15 M KCl solution. The titration was performed starting from pH 11. Using the Yasuda−Shedlovsky Plot,[36] the pK_a values could be calculated and extrapolated to 0% dioxane.

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