Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 3127–3132

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

STRUCTURALLY SIMILAR SMALL MOLECULE PHOTOAFFINITY CCK-A AGONISTS AND ANTAGONISTS AS NOVEL TOOLS FOR DIRECTLY PROBING 7TM RECEPTOR–LIGAND INTERACTIONS

James W. Darrow,[a,*] Elizabeth M. Hadac,[b] Laurence J. Miller,[b] and Elizabeth E. Sugg[c]

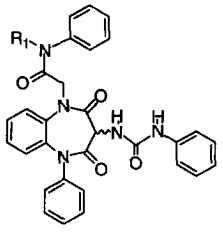
[a]Neurogen Corporation, Branford, CT 06405, U.S.A.; [b]Center for Digestive Diseases, Mayo Clinic, Rochester, MN, U.S.A.; and [c]Department of Medicinal Chemistry, Glaxo Wellcome, 5 Moore Drive, Research Triangle Park, NC 27709 U.S.A.

Received 24 June 1998; accepted 21 September 1998

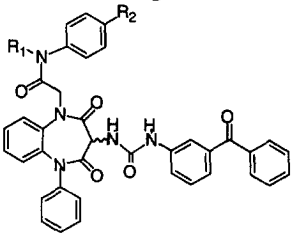
Abstract: Incorporation of photolabile benzoyl (**2a–d**) or trifluoromethyl-3*H*-diazirine (**3a–d**) substituents into 1,5-benzodiazepine ligands did not significantly impair the rat CCK-A binding affinity of either agonists or antagonists. The modified agonist ligands also retained functional potency and efficacy in the rat amylase assay. Despite their strong structural similarity, the SAR of this limited set of compounds suggests that these small molecule antagonists and agonists might differ in their mode of binding to the CCK-A receptor. Preliminary affinity results show that representative agonists and antagonists from these series can be used to efficiently covalently label the CCK-A receptor.

We have recently reported 1,5-benzodiazepine ligands that, with only a minor change in amide substitution from *N*-isopropyl to *N*-methyl, act as either cholecystokinin-A (CCK-A) agonists or antagonists, respectively.[1] Incorporation of photoaffinity labels into these structurally similar 1,5-benzodiazepine agonists and antagonists may allow direct study of the molecular interactions between the ligand and the CCK-A receptor, complementing previous work using peptide photoaffinity ligands.[2–4] In particular, photoaffinity labeling experiments using these ligands would directly address whether the binding of small molecule ligands, especially nonpeptidyl agonists, differs from that of peptide ligands, as well as provide evidence as to whether structurally analogous small molecule agonists and antagonists bind the CCK-A receptor in a similar fashion.

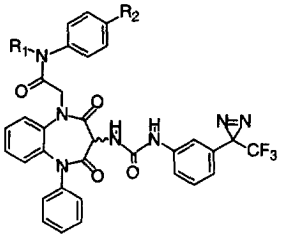
Figure 1



1: R_1 = CH_3 (antagonist)
= *i*Pr (agonist)



2 a: R_1= CH_3, R_2= H
b: R_1= *i*Pr, R_2= H
c: R_1= CH_3, R_2= OCH_3
d: R_1= *i*Pr, R_2= OCH_3



3 a: R_1= CH_3, R_2= H
b: R_1= *i*Pr, R_2= H
c: R_1= CH_3, R_2= OCH_3
d: R_1= *i*Pr, R_2= OCH_3

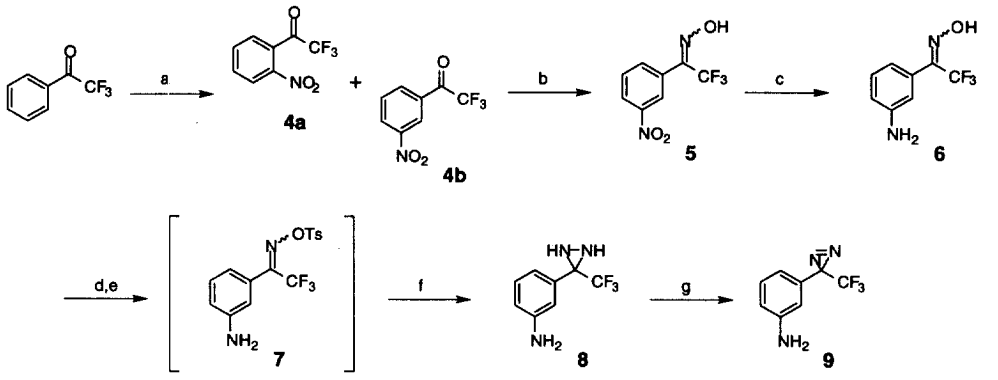
0960-894X/98/$ - see front matter
PII: S0960-894X(98)00548-4

We report here the synthesis, characterization, and in vitro biological evaluation of two series of photoaffinity labels for the CCK-A receptor, based on the previously reported structure **1** (Figure 1).[1] These target ligands, compounds **2** and **3** shown in Figure 1, consist of four antagonist/agonist pairs which differ in both the *N*-1 "trigger" region and in the nature of the photolabile moiety incorporated through the C-3 phenylurea linkage.

Chemistry

The basic strategy involved incorporation of various *meta*-substituted[5] photoaffinity groups in the C-3 phenylurea position of the model compound **1** through a convergent synthetic approach. A wide variety of photoaffinity labels are reported in the literature.[6,7] From this selection, the aryl-3-trifluoromethyl diazirines and the benzophenones were chosen for use in our system, based on chemical stability, low incidence of solvent insertion and their demonstrated ability to readily insert into active site C-H bonds.[6–10] Since our knowledge of the CCK-A receptor binding site for either small molecule antagonist or agonist ligands was limited, we elected to synthesize both photolabels for initial biological testing.

Scheme 1 illustrates the synthetic approach employed for the diazirine **9**, as modified from the general procedure of Brunner and coworkers.[11] Conventional arylnitration of trifluoroacetophenone provided the known compounds **4a,b** as a (35:65) mixture of *ortho*- and *meta*- isomers in near quantitative yield.[6,12] Due to problems with ketone reduction, **4b** was first converted to the more reductively stable oxime **5**,6 then hydrogenated to the *anilino*-oxime **6** under atmospheric pressure.

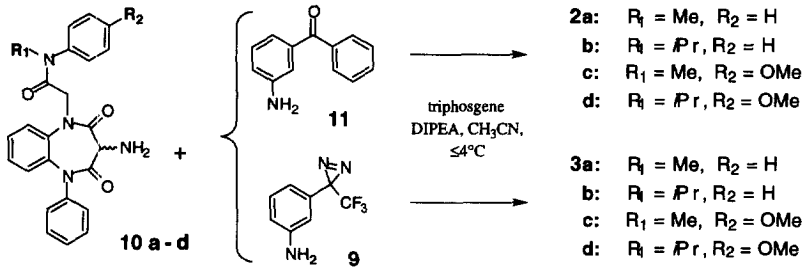


Scheme 1. (a) HNO_3, H_2SO_4, ≤4 °C; (b) NH_2OH•HCl, Pyridine, EtOH, 60 °C; (c) Pd/C, H_2, EtOH, rt; (d) *t*-BuOK, THF, ≤4 °C; (e) THF, TsCl, ≤4 °C; (f) NH_3, CH_2Cl_2, 77 °C; (g) Ag_2O, Et_2O, rt.

The *O*-tosylated species **7** was obtained selectively by pretreatment of the *anilino*-oxime **6** with one equivalent of potassium *t*-butoxide, followed by reaction with tosyl chloride. Reaction of the crude *O*-tosylate with liquid ammonia in the next step led to the diaziridine **8**. Near quantitative yields for the oxidation of the

diaziridine to the diazirine **9** were achieved through use of freshly prepared Ag_2O in diethyl ether.[6] The course of reaction, from oxime to diaziridine to diazirine could be easily monitored by ^{19}F NMR analysis of the crude reaction solution (chemical shifts approximately –67 ppm, –76 ppm, and –65 ppm, respectively).

Syntheses of the four C-3 amines **10a–d** proceeded smoothly, based on previously reported methods.[1,5] During subsequent coupling of **10a–d** with the respective photolabile anilines **9** and **11**, symmetric urea formation was minimized by first reacting the *anilino*-photophore with triphosgene at low temperature, then treating the resulting intermediates in situ with the four different *N*-alkylated-C-3 aminobenzodiazepines (**10a–d**).



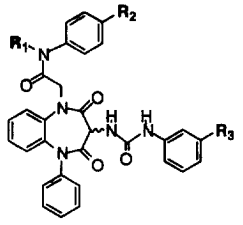
Scheme 2.

Biology

Analogs **2a–d** and **3a–d** were first evaluated for their CCK-A receptor binding affinity on membrane preparations isolated from rat CCK-A receptor-bearing cell line, CHO-CCKR.[13] The IC_{50} values for the photoaffinity ligands **2a–d** and **3a–d**, as well as for reference compounds **1b–d** and CCK-8, are given in Table 1. Compounds **2a–d** and **3a–d** were subsequently evaluated for their ability to stimulate secretion of amylase from dispersed rat pancreatic acini,[14] using CCK-8 as an agonist control. The EC_{50} value for each compound is provided in Table 1.

For preliminary photoaffinity studies, representative nonpeptidyl agonist and antagonist ligands were radiolabeled with ^{14}C phosgene as described by Scheme 2 to achieve a specific radioactivity of 0.05 Ci/mmol, in preparation for photoaffinity labeling of the CCK receptor. This was accomplished by incubating 10 μM radioligand with the CHO-CCKR membranes in the dark at 30 °C for 180 min. After binding was allowed to reach steady state, the samples were cooled to 4 °C and photolyzed using 3500 lamps for 30 min. Membranes were collected by centrifugation, washed, and solubilized in 1% digitonin for 16 h at 4 °C. Membrane glycoproteins were concentrated with wheat germ agglutinin-agarose and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, with radiolabeled bands detected by autoradiography. CCK receptor that had been photoaffinity labeled using ^{125}I-D-Tyr-Gly-[(Nle28,31)CCK-26-33] was run in an adjacent lane as a control.[20]

Table 1. In Vitro Activity of 1,5 Benzodiazepine Photoaffinity Ligands



No.	Structures R_1	R_2	R_3	CCK-A binding assay[a] IC_{50} (μM)	Amylase assay[b] EC_{50} (μM)	Efficacy (1.0 μM)
CCK-8[c]				0.80 nM[c]	0.014 nM[c]	
1b[d]	-*i*Pr	-H	H	0.25[d]	0.04[d]	83%[d]
1c[d]	-Me	-OMe	H	0.39[d]	n.d.	--
1d[d]	-*i*Pr	-OMe	H	0.05[d]	n.d.	--
2a	-Me	-H	benzoyl	0.29 ± 0.17	--	--
2b	-*i*Pr	-H	benzoyl	0.16 ± 0.05	0.73 ± 0.18	64%
2c	-Me	-OMe	benzoyl	0.35 ± 0.05	i.a.	--
2d	-*i*Pr	-OMe	benzoyl	0.08 ± 0.02	0.46 ± 0.02	68%
3a	-Me	-H	diazirine	0.31 ± 0.04	i.a.	--
3b	-*i*Pr	-H	diazirine	1.30 ± 0.60	0.93 ± 0.07	47%
3c	-Me	-OMe	diazirine	0.41 ± 0.02	i.a.	--
3d	-*i*Pr	-OMe	diazirine	0.44 ± 0.07	0.60 ± 0.06	75%

[a]Binding affinity for rat CCK-A receptors; concentration of ligand that displaced 50% of ^{125}I-D-Tyr-Gly-[(Nle28,31)CCK-26-33] from membrane preparations isolated from rat CCK-A receptor-bearing cell line, CHO-CCKR, ± SD; 3 determinations.[13] [b]Functional activity based on the ligand's ability to stimulate dispersed rat pancreatic acini to secrete amylase into the medium; EC_{50}, concentration at which 50% of the maximal secretion (normalized to % of CCK-8 maximum activity) was observed ± SD; 3 determinations; n.d. = not determined; i.a. = no activity detected at maximum concentration of ligand used.[14,15] [c]Ref 16. [d]Data provided for comparison.

Results

Substitution at R_1: Substitution of either photolabel was well tolerated, based on receptor binding affinity (Table 1). Similar to previously reported SAR for the competitive antagonist **1**,[1] the methyl compounds competitively displace the radiolabeled agonist peptide and bind the rat CCK-A receptor, yet do not exhibit measurable agonist activity. The binding affinities for photolabile ligands with R_1 = methyl (**2a**, **2c**, **3a**, **3c**) are similar to the affinity for the antagonist standard, **1c**, while the affinities for the ligands with R_1 = isopropyl (**2b**, **2d**, **3b**, **3d**) are comparable to the affinities for the corresponding agonist standards, **1b** and **1d**. Only the isopropyl compounds stimulated the release of amylase from rat pancreatic acini.

Substitution at R_2: Incorporation of the *p*-methoxy substituent in place of hydrogen at R_2 enhances binding affinity for the isopropyl analogs (**2d**, **3d**) but not the methyl analogs (**2c**, **3c**). In the amylase assay, *p*-methoxy substitution also increases potency for both benzophenone and diazirine agonists, but only significantly increases efficacy (47% to 75%, at 1 μM) for the diazirine agonists (**2d**, **3d**). Benzophenone agonist efficacy is not enhanced by *p*-methoxy substitution at R_2, (64% to 68%, at 1 μM).

Substitution at R_3: The nature of the phenylurea substituent (*m*-benzoyl or *m*-diazirine) influences the binding affinity of the agonists. Although the benzophenone agonists (**2b, 2d**) and the corresponding standards (**1b, 1d**) bind the receptor with similar affinity, the diazirine agonists (**3b, 3d**) bind 5- to 10-fold worse than either the standards or the corresponding benzophenone agonists. Phenylurea substitution did not affect the binding affinity of the *N*-methyl analogs, as benzophenones (**2a, 2c**), diazirines (**3a, 3c**), and the standard (**1b**) all bind equally well to the receptor.

Photoaffinity labeling: Each of the photolabile compounds effectively affinity labeled the CCK receptor, migrating at Mr = 85,000–95,000 on the gel, as shown in a representative autoradiograph in Figure 2.

Discussion and Conclusions

Previously, parallel modifications of the substitution at the N-1 position for benzodiazepine ligands had similar effects on CCK-A binding for both 1,5-benzodiazepine agonists and 1,4-benzodiazepine antagonists,[1] suggesting that small molecule agonists and antagonists (including the known 1,4-benzodiazepine CCK-A antagonist devazepide, MK329)[17] might bind the CCK-A receptor in an analogous fashion. Additionally, modifications of the C-3 substituent of both the 1,5- and 1,4-benzodiazepine agonist and antagonist ligands had similar effects on CCK receptor subtype selectivity.[5]

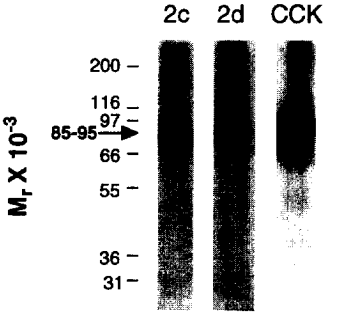


Figure 2. Shown is a representative autoradiograph of a membrane proteins affinity labeled with radiolabeled forms of SDS-polyacrylamide gel used to separate the CHO-CCKR cell nonpeptidyl antagonist **2c**, nonpeptidyl agonist **2d**, and peptide analogue of CCK.[20] All covalently labeled the CCK receptor, migrating at Mr = 85,000–95,000.

The photolabile agonists (**2b, 2d, 3b, 3d**) are sensitive to both phenylurea substitution as well as incorporation of the *p*-OMe in the R_2 position. In contrast, these modifications do not affect the binding of the photolabile 1,5-benzodiazepine antagonists (**2a, c** or **3a, c**). Thus, despite the very high degree of structural similarity between the agonist and antagonist structures, it is possible that the mode of binding to the CCK-A receptor differs for these small molecule antagonists and agonists. Mutagenesis experiments with the angiotensin AT_1 receptor and the recently reported nonpeptidyl partial agonist (L-162,313)[18] provide some precedent for this possibility.[19]

For both antagonists and agonists, incorporation of benzoyl (**2a–d**) or diazirine (**3a–d**) substituents in the *meta*-phenylurea position did not significantly impair binding to the rat CCK-A receptor. More importantly, photolabel incorporation into the *N*-isopropyl compounds did not impair either agonist efficacy or potency in the rat amylase assay.

Preliminary photoaffinity data indicates the ability of representative agonists and antagonists from these series to bind, and subsequently covalently label the CCK-A receptor. Although additional, more detailed, photoaffinity labeling experiments are underway, early results demonstrate that we have successfully developed a unique set of pharmacological probes for evaluating ligand-7TM receptor interactions based on the recently described 1,5-benzodiazepine CCK-A agonists and antagonists.[1] Importantly, these novel compounds include the first examples of nonpeptidyl full agonist ligands which can be used for photoaffinity labeling of nonopioid 7TM receptors. These photoaffinity ligands are also the first reported structurally similar small molecule agonist/antagonists pairs that can be used to directly probe 7TM receptor–ligand interactions.

Acknowledgments: We thank Dr. Jerzy Szewczyk, Dr. Gregory Lowen and Ron Sherrill for helpful discussions. We gratefully acknowledge the financial support of the NIH (DK 32878) and Glaxo Wellcome, Inc. for post-doctoral support of this work.

References

1. Aquino, C. J.; Armour, D. R.; Berman, J. M.; Birkemo, L. S. R.; Carr, A. E.; Croom, D. K.; Dezube, M.; Dougherty, R. W., Jr.; Ervin, G. N.; Grizzle, M. K.; Head, J. E.; Hirst, G. C.; James, M. K.; Johnson, M. F.; Miller, L. J.; Queen, K. L.; Rimele, T. J.; N., S. D.; Sugg, E. E. *J. Med. Chem.* **1996**, *39*, 562.
2. Powers, S. P.; Fourmy, D.; Gaisano, H.; Miller, L. J. *J. Biol. Chem.* **1988**, *263*, 5295.
3. Klueppelberg, U. G.; Gaisano, H. Y.; Powers, S. P.; Miller, L. J. *Biochemistry* **1989**, *28*, 3463.
4. Miller, L. J. *Yale J. Biol. Med.* **1992**, *65*, 441.
5. Hirst, G. C.; Aquino, C.; Birkemo, L.; Croom, D. K.; Dezube, M.; Dougherty, R. W., Jr.; Ervin, G. N.; Grizzle, M. K.; Henke, B.; James, M. K.; Johnson, M. F.; Momtahen, T.; Queen, K. L.; Sherrill, R. G.; Szewczyk, J.; Willson, T. M.; Sugg, E. E. *J. Med. Chem.* **1996**, *39*, 5236.
6. Nassal, M. *Liebigs. Ann. Chem.* **1983**, 1510.
7. Fleming, S. A. *Tetrahedron*, **1995**, *51*, 12479.
8. Brunner, J.; Senn, H.; Richards, F. M. *J. Biol. Chem.* **1980**, *255*, 3313.
9. Dorman, G.; Prestwich, G. D. *Biochem.* **1994**, *33*, 5661.
10. Shih, L. B.; Bayley, H. A. *Anal. Biochem.* **1985**, *144*, 132.
11. Brunner, J.; Semenza, G. *Biochem.* **1981**, *20*, 7174.
12. Stewart, R.; Van der Linden, R. *Can. J. Chem.* **1960**, *38*, 399.
13. Pearson, R. K.; Powers, S. P.; Hadac, E. M.; Gaisano, H.; Miller, L. J. *Biochem. Biophys. Res. Commun.* **1987**, *147*, 346.
14. Lutz, M. P.; Sutor, S. L.; Abraham, R. T.; Miller, L. J. *J. Biol. Chem.* **1993**, *268*, 11119.
15. Ceska, M.; Birath, K.; Brown, B. A. *Clin. Chim. Acta.* **1969**, *26*, 437.
16. Gaisano, H. Y.; Klueppelberg, U. G.; Pinon, D. I.; Pfenning, M. A.; Powers, S. P.; Miller, L. J. *J. Clin. Invest.* **1989**, *83*, 321.
17. Evans, B. E.; Bock, M. G.; Rittle, K. E.; Dipardo, R. M.; Whitter, W. L.; Veber, D. L.; Anderson, P. S.; Freidinger, R. M. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4918.
18. Kivlighn, S. D.; Huckle, W. R.; Zingaro, G. J.; Rivero, R. A.; Lotti, V. J.; Chang, R. S. L.; Schorn, T. W.; Kevin, N.; Johnson, R. G., Jr.; Greenlee, W. J.; Siegl, P. K. S. *Am. J. Physiol.* **1995**, *268*, R820.
19. Perlman, S.; Schambye, J. T.; Rivero, R. A.; Greenlee, W. J.; Hjorth, S. A.; Schwartz, T. W. *J. Biol. Chem.* **1995**, *270*, 1493.
20. Hadac, E. M.; Pinon, D. I.; Ji, Z.; Holicky, E. L.; Henne, R. M.; Lybrand, T. P.; Miller, L.J. *J. Biol. Chem.* **1998**, *273*, 12988.