Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 3327–3330

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Structure Activity Studies of a Novel Cytotoxic Benzodiazepine

Anthony Boitano,[a,†] Cory D. Emal,[a,†] Francesco Leonetti,[b,†] Neal B. Blatt,[a] Thomas A. Dineen,[a] Jonathan A. Ellman,[b] William R. Roush,[a] Anthony W. Opipari[a,*] and Gary D. Glick[a,*]

[a]*Department of Chemistry, University of Michigan, 930 North University Avenue, Ann Arbor, MI 48109-1055, USA*
[b]*University of California—Berkeley, 724 Latimer Hall, Berkeley, CA 94720-1460, USA*

Received 7 April 2003; accepted 15 May 2003

Abstract—Analogues of Bz-423, a pro-apoptotic 1,4-benzodiazepine with potent activity in animal models of systemic lupus erythematosus and rheumatoid arthritis, have been designed, synthesized, and evaluated in cell-culture assays. The results of these experiments have defined the structural elements of this new cytotoxic agent required for activity.

Benzodiazepines such as diazepam and clonazepam are among the most widely studied class of drugs. These compounds exert an anxiolytic effect by increasing γ-aminobutyric acid (GABA) channel gated chloride flux in neural cells.[1] Some benzodiazepines along with isoquinolinecarboxamides like 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195), also bind to a 'peripheral benzodiazepine receptor' (PBR). The PBR is an 18 kDa transmembrane protein located primarily in mitochondria and is distributed in a wide variety of tissues and organs.[2] This protein is one component of an assembly known as the mitochondrial permeability transition pore complex, which serves to regulate the release of pro-apoptotic factors (e.g., cytochrome *c*) in response to various stimuli such as DNA damaging agents. Although nanomolar ligands of the PBR including PK11195 and 4-chlorodiazepam can influence cell survival and proliferation, these effects do not seem to result from binding to this receptor.[2]

We have recently described a 1,4-benzodiazepine (Bz-423, Fig. 1) that is extremely effective at treating disease in two of the most clinically relevant animal models of systemic lupus erythematosus (SLE)[3,4] and a model of rheumatoid arthritis.[4] Unlike current drugs used for lupus (e.g., cyclophosphamide with prednisone), at its therapeutic dose Bz-423 is neither myeloablative nor adversely immunosuppressive. Bz-423 alters disease progression by selectively inducing apoptosis of activated (autoreactive) B and T cells. Incubation of cells with Bz-423 provokes a rapid increase in intracellular superoxide (O_2^-) from mitochondria. This reactive oxygen species (ROS) is an upstream signal that initiates apoptosis. Pre-treating cells with agents that either block the formation of Bz-423-induced O_2^- or scavenge free radicals attenuates the death cascade, demonstrating that cell killing by Bz-423 depends on O_2^-.[3] Although the mechanistic basis for the observed selectivity has not yet been fully elucidated, lupus lymphocytes have less

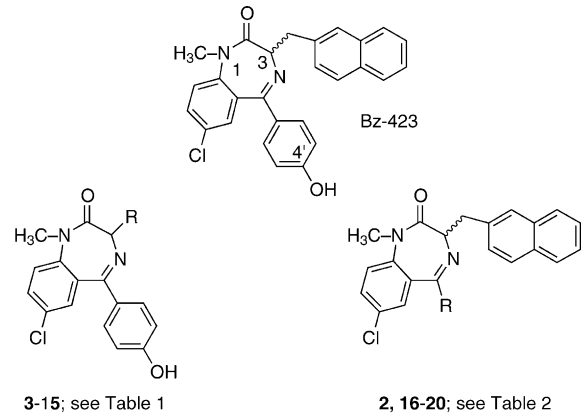


Figure 1. Structure of Bz-423 and analogues. Bz-423 does not bind to the GABA channel and the affinity for the PBR is >1 μM.[3]

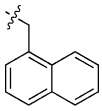
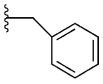
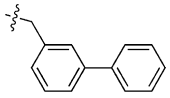
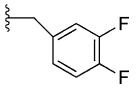
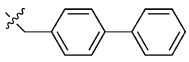
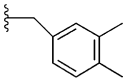
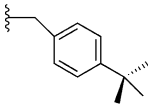
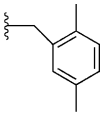
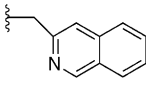
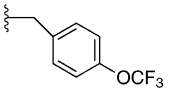
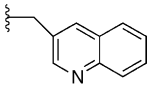
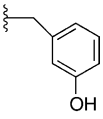
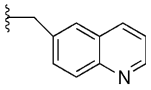
*Corresponding authors. Tel.: +1-734-764-4548; fax: +1-734-615-8902; e-mail: gglick@umich.edu
†Co-first authors.

0960-894X/$ - see front matter © 2003 Published by Elsevier Ltd.
doi:10.1016/S0960-894X(03)00683-8

glutathione and increased ROS relative to normal cells.[5] Thus, their vulnerability may result from an additive effect of Bz-423-induced O_2^- and endogenously generated ROS, such that radicals from all sources combine to overwhelm the limited reducing potential of autoimmune lymphocytes and trigger apoptosis.

Based on these properties of Bz-423, we synthesized a range of derivatives to probe the structural elements of this novel compound required for activity.[6] Replacing the *N*-methyl group or chlorine with a hydrogen atom did not diminish the lymphotoxic activity of Bz-423 against immortalized Ramos B cells in culture (the EC_{50} of Bz-423 is 4 μM; see Fig. 1). Similarly, the enantiomers of Bz-423 were equipotent, which indicates that the interaction between Bz-423 and its molecular target involves two-point binding. In contrast to these data, replacing either the naphthylmethyl group (analogue **1**) or the phenolic hydroxyl group with a hydrogen atom (analogue **2**) abolished all cytotoxic activity ($EC_{50} > 80$ μM; Fig. 2). Based on these observations we focused on changes to the C3 and C′4 positions (Tables 1 and 2). Replacing the 2-naphthylalanine group with a 1-naphthylalanine unit (**3**) had little effect on cell killing. Similarly, replacing the naphthalene moiety with other hydrophobic groups of comparable size (**4–6**) had little effect on the cytotoxic properties of Bz-423. By contrast, quinoline derivatives **7–9** were each less potent than Bz-423. These data suggest a preference for a hydrophobic substituent within the binding site of the molecular target for Bz-423. Smaller C3 substituents (**10–13**) were only somewhat less potent than Bz-423, whereas compounds with aromatic groups containing oxygen were significantly less cytotoxic (**14** and **15**). Although the binding site has yet to be fully mapped with respect to stereoelectronic complementary, these data clearly indicate that a bulky, hydrophobic, aromatic substituent is needed for optimal activity (non-polar aliphatic C3 substituents are not active; unpublished data).

Table 1. Potency of Bz-423 derivatives with altered C3 side chains[a]

C3 side chain	Compd	EC_{50} (μM)[a]	C3 side chain	Compd	EC_{50} (μM)
	**3**	5		**10**	12
	**4**	4		**11**	10
	**5**	7		**12**	7
	**6**	3		**13**	7
	**7**	10		**14**	30
	**8**	11		**15**	22
	**9**	13			

[a]Cell death was assessed by culturing Ramos B cells in the presence of each compound in a dose–response fashion. Cell viability was measured after 24 h by propidium iodide exclusion using flow cytometry. In this assay, the EC_{50} for PK11195, diazepam, and 4-Cl-diazepam is > 80 μM.

Placing a methyl group ortho to the phenolic hydroxyl group (**16**) did not alter the activity of Bz-423, whereas moving the hydroxyl to the C′3 position (**17**) decreased potency 2-fold (Table 2). In contrast, replacing the phenolic hydroxyl group with chlorine (**18**) or azide (**19**), or methylating the phenol (**20**), nearly abolished all the cytotoxic activity of Bz-423. These data indicate that a hydroxyl group positioned at the C′4 carbon is required for optimal activity, possibly by making a critical contact with the physiologically relevant target of Bz-423. However, molecules possessing a phenolic substructure can also act as alternate electron carriers within the mitochondrial respiratory chain (MRC).[7] Such agents accept an electron from MRC enzymes and transfer it back to the chain at points of higher reducing

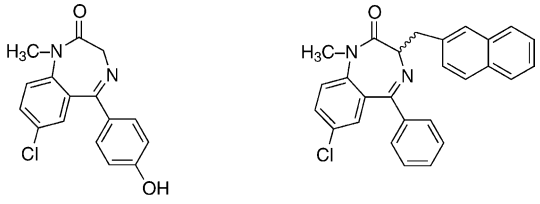
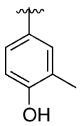
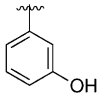
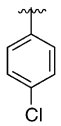
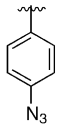
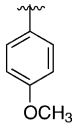


Figure 2. Bz-423 derivatives replacing either the naphthylalanine or phenolic hydroxyl group with a hydrogen atom.

Table 2. Potency of Bz-423 derivatives with changes to the C′4 or C′3 positions[a]

Aryl Group					
Compd	**16**	**17**	**18**	**19**	**20**
EC_{50}	3	6	> 80	> 80	> 80

[a]Cell death was assessed as described in Table 1.

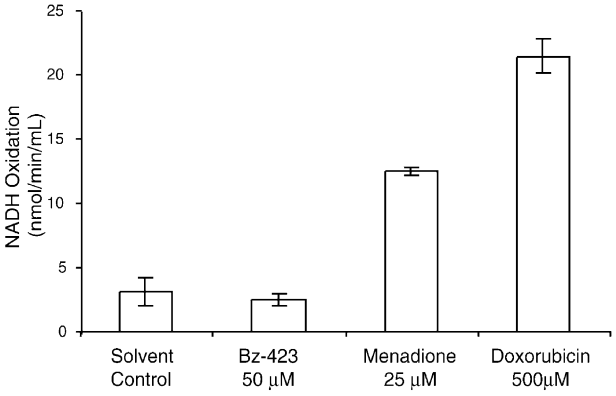


Figure 3. NADH oxidation assay using beef heart submitochondrial particles. Bz-423 was used at $>10\times$ the EC_{50}. Similar results were obtained with Bz-423 using NADPH as a substrate (data not shown).

Table 3. Inhibition of cell death by Bz-423 derivatives with FK506 and **20**

Compd	% Inhibition with FK506 (1 μM)	% Inhibition with **20** (20 μM)
Bz-423	81	80
1	ND	ND
2	ND	ND
3	72	67
4	77	81
5	74	69
6	91	55
7	42	0
8	39	4
9	20	0
10	4	0
11	59	0
12	78	29
13	80	23
14	17	0
15	14	20
16	78	87
17	70	0
18	ND	ND
19	ND	ND
20	ND	ND

ND, not determined.

potential. This type of 'redox cycling' consumes endogenous reducing equivalents (e.g., glutathione) along with pyrimidine nucleotides and results in cell death. To better ascertain the role of the phenolic hydroxyl group, we determined if Bz-423 redox cycles in the presence of beef heart submitochondrial particles using standard NADH and NADPH oxidation assays as previously described.[8] Unlike the positive controls (doxorubicin and menadione), Bz-423 does not lead to substrate oxidation, which strongly suggests that it does not redox cycle (Fig. 3). Collectively, therefore, our data suggest that the difference in potency between Bz-423 and **18**–**20** results from removing an interaction that mediates target binding *and* activity.

Cells rapidly produce O_2^- in response to Bz-423 and blocking this signal (e.g., by inhibiting ubiquinol cytochrome *c* reductase, which is the enzyme within the MRC that produces ROS in response to Bz-423) prevents apoptosis.[3] To determine if the derivatives listed in Tables 1 and 2 kill cells in a manner analogous to Bz-423, presumably as a result of binding to a common molecular target, we examined the ability of FK506, micromolar amounts of which effectively inhibit ubiquinol cytochrome *c* reductase, to protect against cell death.[3] Substantial inhibition by FK506 ($\geq$60%)[9] was only observed for **3**–**6**, **12**, **13**, **16**, and **17**, which are the compounds with hydrophobic C3 side chains larger than phenyl (Table 3). With the exception of **17**, cell death induced by both Bz-423 and the group of compounds blocked by FK506, was also inhibited by pretreating cells with **20** (20 μM). Compound **20** blocks the cytotoxic action of Bz-423 in a dose dependent fashion presumably by acting as a competitive inhibitor.[9,10] These data suggest that Bz-423, **3**–**6**, **12**, **13**, **16**, bind the same site within the target protein and induce apoptosis through a common mechanism. In contrast to these data, compound **20** had no effect on blocking the cytotoxic activity (inhibition $\leq$10%) of the other benzodiazepines listed in Table 1. The other compounds apparently do not bind at this site and may induce a death response through a different pathway.

In summary, we have presented a structure–activity study for a family of new 1,4-benzodiazepines with profound therapeutic activity in the animal models of human lupus and autoimmune arthritis. Analysis of the analogues in Tables 1 and 2 indicate that the aromatic side chain and the phenolic hydroxyl group in the C'4 position are key elements for target binding and cytotoxic activity. In addition, competition experiments with **20** revealed which aromatic side chains promote binding to the molecular target of Bz-423. Present studies are directed at identifying the mitochondrial target for these benzodiazepines to advance both drug discovery efforts and additional mechanistic experiments.

Acknowledgements

This research was supported by NIH grants AI-47450 and GM-42168 (to G.D.G.), GM-50353 (to J.A.E.), and AI 35709 (to W.R.R). The authors thank J. Patchett for the synthesis of **18**.

References and Notes

1. Korpi, E. R.; Grunder, G.; Luddens, H. *Prog. Neurobiol.* **2002**, *67*, 113.
2. Beurdeley-Thomas, A.; Miccoli, L.; Oudard, S.; Dutrillaux, B.; Poupon, M. F. *J. Neuroncol.* **2000**, *46*, 45.
3. Blatt, N. B.; Bedarski, J. J.; Warner, R.; Leonetii, F.; Johnson, K. M.; Boitano, A.; Johnson, K. J.; Yung, R.; Richardson, B. C.; Ellman, J. A.; Opipari, A. W.; Glick, G. D. *J. Clin. Invest.* **2002**, *10*, 1123.
4. Bedarski, J. J.; Warner, R.; Rao, T.; Leonetii, F.; Johnson, K. J.; Yung, R.; Richardson, B. C.; Ellman, J. A.; Opipari, A. W.; Glick, G. D. *Arthritis Rheum.* **2003**, *48*, 757.
5. Gergely, P., Jr.; Grossman, C.; Niland, B.; Puskas, F.; eupane, H.; Allam, F.; Banki, K.; Phillips, P. E.; Perl, A. *Arthritis Rheum.* **2002**, *46*, 175.
6. The *R* and *S* isomers of Bz-423 were synthesized as described by Bunnin: Bunin, B. A.; Plunkett, M. J.; Ellman, J. A.

Proc. Natl. Acad. Sci. U.S.A. **1994**, *91*, 4708. All other compounds were racemic and were prepared according to Kim:\05 Kim, K.; Volkman, S. K.; Ellman, J. A. *J. Braz. Chem. Soc.* **1998**, *9*, 375. All compounds afforded NMR and MS data consistent with the proposed structures.
7. Wallace, K. B.; Starkov, A. A. *Annu. Rev. Pharmacol.* **2000**, *40*, 353.
8. Shimada, H.; Hira, K.; Samamura, E.; Pan, J. *Arch. Biochem. Biophys.* **1998**, *75*, 351.
9. In these experiments, the test benzodiazepines were used at their respective EC_{50} values.
10. **20** was used at 20 μM because this concentration provides maximal inhibition of Bz-423.