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Identification of cytotoxic, T-cell-selective 1,4-benzodiazepine-2,5-diones

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Abstract—A family of 1,4-benzodiazepine-2,5-diones (BZDs) has been synthesized and evaluated against transformed B- and T-cells for lymphotoxic members. A large aromatic group on the C3 position is critical for cytotoxicity. When the C3 moiety contains an electron-rich heterocycle, the resulting BZDs have sub-micromolar potency and are selective for T-cells. Cell death is consistent with apoptosis and does not result from inhibition of the mitochondrial F_oF_1-ATPase, which is the molecular target of recently reported cytotoxic 1,4-benzodiazepines. Collectively, these studies begin to characterize some of the structural elements required for the activity of a novel family of T-cell-selective lymphotoxic agents.

Certain autoimmune diseases and hematological malignancies are associated with aberrant survival and expansion of B- or T-cell populations.[1] Current therapies for these disorders generally include cytotoxic drugs whose mechanisms of action frequently involve DNA damage.[2] Hence, selectivity of these drugs relies on the differential ability of diseased and healthy cells to tolerate and repair drug-induced DNA damage and is often limited.

Bz-423 is a pro-apoptotic 1,4-benzodiazepine with potent activity in two clinically relevant animal models of systemic lupus erythematosus (Fig. 1).[3,4] Bz-423 binds to the oligomycin sensitivity conferring protein component of the mitochondrial F_oF_1-ATPase.[5] Bz-423 inhibits the enzyme, which produces a state 3 to state 4 transition within the mitochondrial respiratory chain (MRC), ultimately resulting in production of superoxide (O_2^-) from MRC complex III. This reactive oxygen species acts as an upstream signal initiating a tightly regulated apoptotic process.[5] Previous studies revealed that a hydrophobic aromatic substituent at C3 along with the phenolic hydroxyl group is required for the cytotoxic activity of Bz-423.[6]

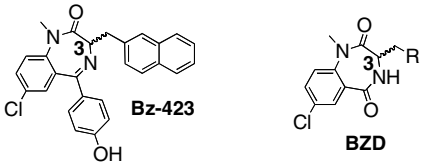

Figure 1. Structure of Bz-423 and 14,-benzodiazepine-2,5-diones.

As part of efforts to further define the elements of Bz-423 required for inhibiting the F_oF_1-ATPase, a series of 1,4-benzodiazepine-2,5-diones (BZDs) with aromatic substitution at C3 were synthesized (Fig. 1; Table 1). Despite the absence of a phenolic hydroxyl group, most of these compounds were quite cytotoxic. BZD-induced cytotoxicity is characterized by cell shrinkage, cytoplasmic vacuolization, membrane blebbing, and DNA fragmentation (i.e., appearance of hypodiploid DNA) consistent with apoptosis (Supplementary Figure 1). Many of these compounds displayed greater lymphotoxic activity against Jurkat T-cells (a transformed T-lymphoblast line derived from peripheral blood leukemia) compared to Ramos B-cells (a B-lymphoblast line

Keywords: Apoptosis; Autoimmunity benzodiazepine; Cancer; Mitochondria.
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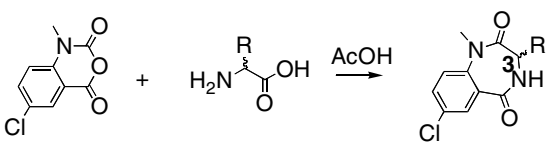
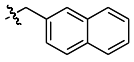
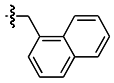
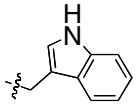
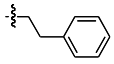
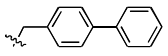
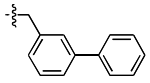
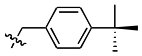
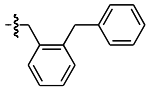
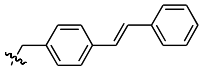
Table 1. Potency of BZDs with substitution at the C3 position



Compound	R	EC_{50} (μM)	
		Ramos B-cells	Jurkat T-cells
1		10.7	3.0
2		12.6	20.5
3		9.2	26.2
4		>100	>80
5		>100	>80
6		6.2	2.3
7		19.1	5.4
8		16.2	8.2
9		18.6	17.6
10		50.3	7.5

Cell death was measured by propidium iodide (PI) staining as described previously.[4] Data are means of three experiments with a standard deviation of ±3%.

derived from Burkitt's lymphoma; Table 1). By contrast, the EC_{50} of Bz-423 for Ramos B- and Jurkat T-cells is quite similar (~7.0 μM).

Based on these properties, we synthesized a series of derivatives to probe the structural elements necessary for T-cell-selective cytotoxicity by condensing *N*-methyl 5-chloroisatoic anhydride with glycine derivatives in refluxing acetic acid (Table 1; ^{1}H NMR and MS data for all compounds are presented as Supplementary Data). [7]

First, the naphthyl moiety was replaced with other hydrophobic groups of comparable size (**2**, **3**), but which are oriented differently. Compared to **1**, these substitutions had relatively small effects on activity in Ramos B-cells, but greatly increased the EC_{50} in Jurkat T-cells. In addition, reducing the size of the C3 substituent (**4**, **5**) resulted in a dramatic decrease in activity against both cell types. Substitution with a *p*-biphenyl moiety (**6**) improved activity in both cell types, whereas substitution with *m*-biphenyl (**7**) or *p*-phenyl *tert*-butyl (**8**) moieties or incorporation of carbon linkers between the two rings (**9**, **10**) failed to improve activity or selectivity. These data reveal that the size of the C3 substituent is critical for the increase in T-cell-selective cytotoxicity relative to **1**. Moreover, they suggest that it may be possible to optimize potency and selectivity by further substitution of the *p*-biphenyl or 2-naphthylene C3 side chains.

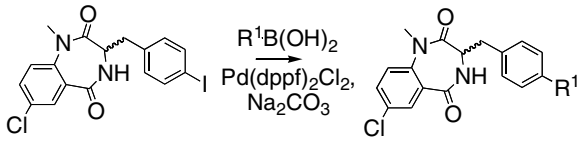
To further explore the structural and electronic requirements of the *p*-biphenyl substituent (**6**) at C3, a range of substituted biphenyls were prepared by Suzuki coupling of aryl halides with commercially available boronic acids (Table 2).[7,8] In the first group of derivatives (**11**–**16**), a methyl group or chlorine atom was substituted at the 2′, 3′, or 4′ positions. Although selectivity was lost, the data indicated that certain substitutions at the 3′ position improve potency (**12**, **15**).

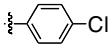
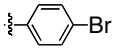
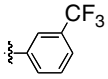
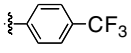
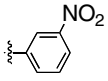
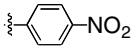
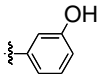
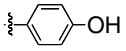
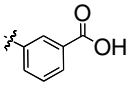
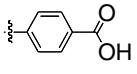
Substitution of a bromine atom (**17**, **18**) at the 3′ or 4′ positions decreased activity and selectivity relative to **6**. Placement of a trifluoromethyl group (**19**, **20**) at the same positions resulted in moderate, equipotent cytotoxicity against B- and T-cells. Substitution of an electron-withdrawing nitro group at the 3′ position (**22**) improved potency against Jurkat T-cells while maintaining 3-fold selectivity over Ramos B cells. Substitution of a nitro group at the 4′ position (**23**) afforded activity and selectivity similar to **1**, whereas 2′ substitution (**21**) ablated activity in both cell lines.

The improvement in potency with retention of selectivity that was achieved by substitution of either the 3′ or 4′ positions prompted incorporation of hydroxyl (**24**, **25**) and carboxyl groups (**26**, **27**) at these positions. While the hydroxyl analogs both displayed significant T-cell cytotoxicity and selectivity similar to those of **6**, the carboxyl substituents greatly diminished activity against both cell types. Because of the improved potency and selectivity observed with the electron-rich hydroxyl substitutions, pyridine (**28**, **29**) and furan (**30**) analogs were prepared. Both pyridines achieved sub-micromolar potency against Jurkat T-cells, and selectivity was maximized when the nitrogen was in the 3′ position. Finally, substitution of the terminal phenyl ring with a furan produced the most potent compound **30**, albeit with slightly diminished selectivity relative to pyridine **28**.

Given the similarity between the structures of the cytotoxic BZDs reported here and compounds like Bz-423, we determined if the pyridine-substituted BZDs (**28**, **29**) inhibit the F_oF_1-ATPase or generate O_2^- similar to Bz-423. The pyridine-substituted analogs (**28**, **29**) were selected as representative because they are among the most potent and T-cell-selective BZDs. Neither **28** nor **29** inhibited the ATP synthase activity of the F_oF_1-ATPase nor induced a cellular O_2^- response (Fig. 2). This indicates that these BZDs have a different molecular target and apoptotic mechanism than the Bz-423 family of benzodiazepines. In support of this

Table 2. Activity of BZDs with various R^1 groups



Compound	R^1	EC_{50} (μM)		Selectivity
		Ramos B-cells	Jurkat T-cells	
11		6.7	5.4	1.2
12		2.8	3.1	0.9
13		6.9	3.6	1.9
14		10.3	8.3	1.2
15		1.5	2.0	0.8
16		6.5	6.5	1.0
17		9.2	4.8	1.9
18		>20	9.8	>2
19		4.0	4.5	0.9
20		4.8	5.2	0.9
21		>20	>20	n/a
22		4.2	1.5	2.8
23		10.0	3.8	2.6
24		4.0	1.5	2.7
25		6.0	1.5	4.0
26		>20	>20	n/a
27		31.8	15.1	2.1
28		6.0	0.6	10.0

(*continued on next page*)

Table 2 (*continued*)

Compound	R^1	EC_{50} (μM)		Selectivity
		Ramos B-cells	Jurkat T-cells	
29		3.0	0.5	6.0
30		2.0	0.3	6.5

Cell death was measured by propidium iodide (PI) staining as described previously.[4] Data are means of three experiments with a standard deviation of ±3%. Selectivity is defined as Ramos EC_{50}/Jurkat EC_{50}, and is listed as not applicable (n/a) when EC_{50} values for both cell types are >20 μM.

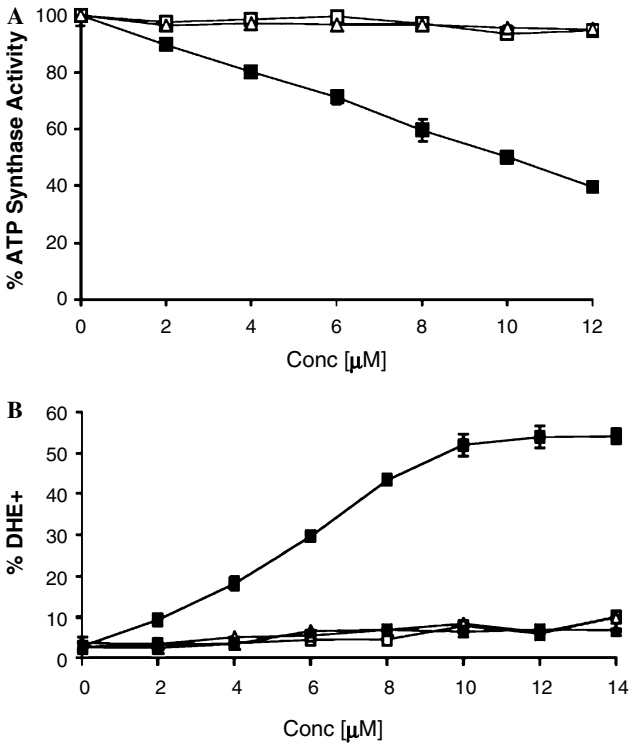


Figure 2. (A) Bovine sub-mitochondrial particles were treated with Bz-423 (closed squares), **28** (open squares), or **29** (open triangles) and rates of ATP synthesis were determined using an $NADP^+$-coupled assay as described previously.[5] (B) Jurkat T-cells incubated with Bz-423 (closed squares), **28** (open squares), **29** (open triangles), or clonazepam (a benzodiazepine that does not produce O_2^-; closed triangles) for 1 h and O_2^- production was measured by flow cytometric analysis of dihydroethidium (DHE) fluorescence as described previously.[4]

hypothesis, pretreatment with the antioxidants manganese(III)tetrakis (4-benzoic acid)porphyrin chloride and vitamin E, which prevent Bz-423-induced cell death, failed to block apoptosis induced by **28** and **29** (data not shown).

The benzodiazepine-dione core has been widely used for preparation of biologically active molecules; for example, endothelin receptor antagonists,[9] inhibitors of platelet aggregation,[10] and p53-HDM2 antagonists.[11] Although the BZDs described here share a similar scaffold and biological activity with p53-HDM2 antagonists, several lines of evidence indicate that they are not expected to induce apoptosis by blocking p53-HDM2 binding. First, the SAR of the critical C3 side chains differs between the two classes of compounds.[11] Second, in Ramos B-cells one copy of the p53 gene is deleted and the remaining allele encodes a mutant protein incapable of activating transcription or mediating apoptosis.[12]

T-cell activation and proliferation is dependent on cytokines and cell–cell interactions. As such, experimental therapies for T-cell-mediated disease typically use either soluble receptor decoys or monoclonal antibodies to neutralize these interactions and prevent pathogenic activation and cellular proliferation.[13] In addition, small molecule inhibitors of purine nucleoside phosphorylase (PNP), an enzyme that catalyzes degradation of deoxyguanosine, selectively kill T-cells relative to other lymphoid subsets.[14] We have presented a preliminary structure–activity study for a family of new 1,4-benzodiazepine-2,5-diones, which like PNP inhibitors are selectively cytotoxic against transformed T-cells. Since these BZDs are structurally unrelated to the PNP antagonists, it is unlikely that they have the same molecular mechanism and target. Therefore, these BZDs are promising leads that in a forward chemical genetics fashion may help to identify a new molecular target and mechanism for treatment of T-cell diseases. As such, current efforts focus on improving the potency of this series of compounds as well as identifying the physiologically relevant target and mechanism underlying the T-cell selectivity.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.01.113.

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