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Bioorganic & Medicinal Chemistry Letters 13 (2003) 3181–3184

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Regulation of Gene Expression by Synthetic Dimerizers with Novel Specificity

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Received 13 May 2003; accepted 1 July 2003

Abstract—New synthetic chemical inducers of dimerization, comprising homodimeric FKBP ligands with engineered specificity for the designed point mutant F36V, have been evaluated for inducing targeted gene expression in mammalian cells. Structure–activity studies indicated that high-affinity dimerizers such as AP1903 are ineffective, perhaps due to kinetic trapping of non-productive dimers, whereas lower-affinity molecules, exemplified by AP1889 and AP1966, potently activate transcription.

The use of chemical inducers of dimerization (CIDs), or dimerizers, to control protein–protein interactions is now well established. Proteins of interest are expressed in cells as fusions to ligand-binding domains; addition of a bivalent ligand drives association of the proteins. Since the initial report of FK1012, a semi-synthetic dimer of FK506, and its use to control the association of FKBP fusion proteins,[1] chemical dimerization has been used to create inducible alleles of numerous signaling proteins that are naturally activated by oligomerization.[2] Induced dimerization can also be used to control gene expression, by fusing drug binding domains to the separate DNA-binding and activation domains of a transcription factor: addition of dimerizer reconstitutes an active transcription factor, initiating expression of a gene engineered with an appropriate promoter.[3–5] Dimerizer-induced gene expression is a useful research tool for analyzing gene function, and also has potential therapeutic utility for the pharmacologic control of gene expression in the context of gene therapy.[6]

Previously, we reported the synthesis and structure–activity relationship of a family of bivalent FKBP ligands typified by the homodimeric compound AP1510 (**1a**, Fig. 1).[7,8] These molecules are much simpler than FK1012, are of lower complexity and molecular weight than FK1012, and are fully synthetic. In addition, AP1510 was found to be significantly more potent than FK1012 in inducing Fas-mediated apoptosis of engineered cells, and in activating the expression of target genes.[7]

Despite the advantages of AP1510 and its congeners, their use in vivo is potentially complicated by their interaction with endogenous FKBP molecules, which are ubiquitously expressed in eukaryotic cells, often at high levels. Interaction with endogenous FKBPs could potentially interfere with natural FKBP functions and could also blunt dimerizer potency through the sequestration of dimerizer in FKBP-rich cells, such as erythrocytes. To address this issue, we further modified the compounds to enhance their specificity for engineered fusion proteins using a 'bump and hole' strategy.[9,10] A bulky substituent ('bump') was added to each monomeric unit to sterically disrupt FKBP binding, and a complementary 'hole' was then generated in the binding pocket of the fusion FKBP construct by structure-guided site-directed mutagenesis.

We have reported the successful use of this strategy to generate the dimerizer AP1903 (**1b**) (Fig. 1), which binds with high affinity and >1000-fold selectivity to the Phe36Val (F36V) mutant of FKBP compared to the wild-type protein.[9] AP1903 and its congeners potently activate signaling in cells expressing FKBP-F36V fusion proteins, both in cell culture and in a variety of in vivo settings.[9,11] However, the evaluation of these engineered dimerizers for use in regulated gene expression has not been reported. We describe here the synthesis and structure–activity studies of a series of AP1903 congeners optimized for the regulated transcription of target genes.

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doi:10.1016/S0960-894X(03)00707-8

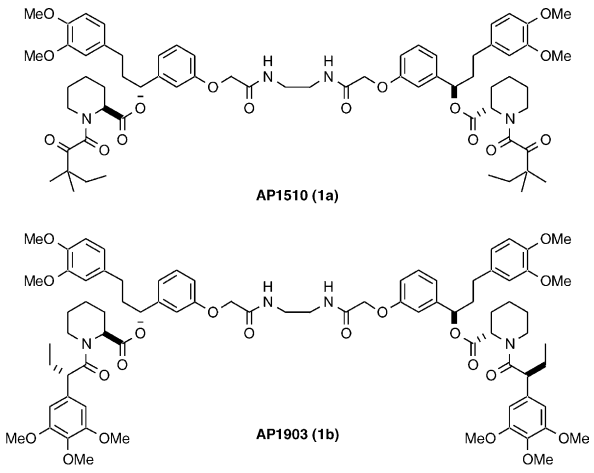


Figure 1. Structures of AP1510 and AP1903.

Chemistry

The general methods used for the preparation of synthetic monomers and dimerizers **1a**–**1k** have been reported.[7,8,10] The synthesis of the urea dimer **1k** is outlined in Scheme 1. Pipecolyl ester **2**[10] was treated with an activated carbamate obtained from reaction of 3-aminopentane with *para*-nitrophenyl chloroformate. The resulting urea was then treated with TFA to remove the *tert*-butyl ester and the resulting carboxylic acid coupled via BOP with a half of an equivalent of ethylenediamine to afford **1k**.

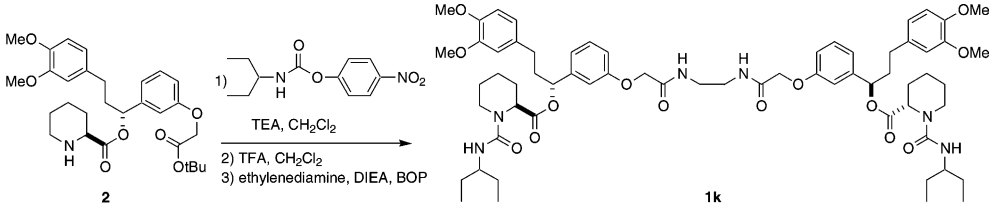
Results

The assay system for determining the ability of AP1903-type bumped dimerizers to activate gene expression[12] is similar to that described previously for the unbumped dimerizer AP1510.[7,8] In short, HT1080 human fibrosarcoma cells are transiently or stably transfected with constructs encoding separate DNA-binding domain and activation domain proteins, each fused to three tandem copies of F36V-FKBP. The cells additionally harbor a SEAP (secreted alkaline phosphatase) reporter gene responsive to the regulated transcription factor, so that productive dimerization of the fusion proteins leads to SEAP production. We tested both transiently and stably transfected cells since these represent two frequent uses of the regulated gene expression system, and differ in the expression levels of the fusion proteins (which are much higher in the transient system). Table 1 presents the transcriptional activity of each dimerizer tested, reported both as an EC50 and a peak expression level as a percentage of that of AP1889 (**1f**), the compound ultimately identified as the most potent in this series. To investigate the relationship between binding affinity and potency, we also determined the relative F36V-FKBP binding affinities of the monomeric FKBP ligands used to assemble dimerizers using a fluorescence polarization (FP) assay.[9]

Consistent with previous assays using the transcriptional system with wild-type FKBP fusion proteins,[7] AP1510 (**1a**) induced much higher levels of transcription than FK1012 in transient assays using the F36V-FKBP 'holed' system (Table 1), despite its weaker affinity (IC_{50} of monomer: 44 nM vs 12 nM). Furthermore in stably transfected cells FK1012 proved completely inactive while AP1510 (**1a**) continued to function as a dimerizer, albeit at somewhat higher concentrations (EC_{50}: 177 nM vs 294 nM). Unexpectedly, we found that the divergence of behavior of FK1012 in transiently versus stably transfected cells was recapitulated by AP1903 (**1b**). Despite exhibiting excellent activity in transiently transfected cells (EC_{50}: 3 nM), AP1903 (**1b**) displayed minimal activity in the stably transfected setting.

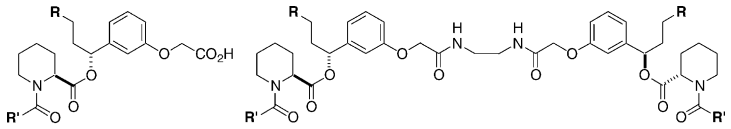
One plausible hypothesis for this behavior of AP1903 (**1b**) is that it is related to the exceptionally high affinity of the compound for F36V-FKBP. The monomer subunit of AP1903 binds to the mutant protein with an IC_{50} of 1.8 nM, but a direct K_d measurement for a fluorescently derivative was determined as $\sim$100 pM.[9] Similarly, FK1012 binds with high affinity to both wild-type and F36V-FKBPs. We wondered whether non-productive dimers, consisting of DNA binding domains (BD–BD) or activation domains (AD–AD), might fail to dissociate readily if induced by high-affinity dimerizers. This would reduce the effective concentration of fusion proteins available to form functional heterodimeric BD–AD complexes. In addition, active BD–AD complexes induced by high-affinity dimerization of multivalent fusion proteins might not dissociate on a timescale compatible with multiple, rapid transcriptional initiation events at a promoter. These problems would both be exacerbated in stably transfected cell lines, in which expression levels of the transcription factor fusion constructs are much lower than in transiently transfected cells. To test this hypothesis, we prepared a number of bumped dimerizers with reduced affinity for F36V-FKBP.

One approach to attenuating the binding affinity of dimerizers for F36V-FKBP is modification of the 3,4-dimethoxyphenyl moiety of AP1903 (**1b**). However, similar to previous observations in the AP1510 series,[8] we found that this modification was far more detrimental to transcriptional activity than to FKBP binding.



Scheme 1. Preparation of **1k**.

Table 1. Affinities of F36V-FKBP12 monomeric ligands and in vitro transcriptional activation activities of synthetic dimerizers



Compd	R	R′	Monomer IC_{50} (nM)[a]	Dimer induced transcription EC_{50} (nM)[b] /% expression[c]	
				Transient	Stable
FK1012			12	189 (39%)	No induction
1a (AP1510)			44	177 (149%)	294 (17%)
1b (AP1903)			1.8	3 (79%)	No induction
1c			5.5	57 (97%)	No induction
1d			15	82 (29%)	No induction
1e			8.2	4 (89%)	No induction
1f (AP1889)			40	4 (100%)	8 (100%)
1g			26	6 (105%)	9 (53%)
1h			1530		
1i			8.2 and 40	3 (115%)	2 (68%)
1j			8.2 and 1530	20 (150%)	10 (7%)
1k (AP1966)			64	11 (138%)	19 (106%)

[a]The IC_{50} is the concentration of monomeric ligand that reduces probe binding by 50% in a fluorescence polarization assay.[9]
[b]The EC_{50} is the concentration of dimerizer that induces 50% peak SEAP reporter protein expression levels.
[c]Where peak expression levels obtained with **1f** (AP1889) = 100%.

Compared to AP1903 (**1b**), replacement with a phenyl (**1c**) or 3-pyridyl (**1d**) group caused 3- and 8-fold reductions in monomer binding affinity to F36V-FKBP respectively, but 20- and 30-fold reductions in EC_{50} (with lower levels of SEAP production) in transiently transfected cells, and no activity in stable cell lines (Table 1). An alternate strategy for reducing affinity for F36V-FKBP is replacement of the ethyl bump of AP1903 (**1b**) with the smaller methyl group. This reduced monomeric binding affinity 5-fold, but the resulting dimerizer **1e** still exhibited transcriptional activities equivalent to those of AP1903 (**1b**).

In an effort to more radically reduce binding affinity, the entire 3,4,5-trimethoxyphenyl 'bottom' portion of AP1903 (**1b**) was replaced by either a phenyl or a 3,4-methylenedioxyphenyl group. Strikingly, this resulted in significant improvements in transcriptional activity. Dimerizer AP1889 (**1f**), whose monomer exhibits a 22-fold reduced affinity for F36V-FKBP, not only proved as effective as AP1903 (**1b**) in transiently transfected cells (with a similar EC_{50}, yet a higher level of protein produced), but also exhibited excellent potency in stably transfected cells, with an EC_{50} of 8 nM.[12] The inter-

mediate affinity 3,4-methylenedioxyphenyl analogue **1g**, which exhibits a 14-fold reduced affinity for F36V-FKBP, similarly proved as effective as AP1889 (**1f**), although induced expression levels in stably transfected cells were lower. All the dimerizers remained selectively active on cells expressing F36V-FKBP fusion proteins compared to wild-type FKBP fusions, demonstrating that the modifications did not affect the binding specificity of the engineered 'bump' (data not shown).

With the availability of a dimerizer able to induce transcription in stably transfected cells, we investigated the activity of a heterodimeric compound **1i** incorporating one monomer unit of AP1889 (**1f**) and one of AP1903 (**1b**). We speculated that if slow dissociation of dimerized protein complexes accounts for AP1903's poor activity, replacement of even one monomer with a weaker binding moiety should still improve potency, since non-productive homodimeric species could be converted to productive ones by dissociation of only one fusion protein followed by rebinding of another. Indeed, **1i** proved to be more potent than either AP1903 (**1b**) or AP1889 (**1f**) in both transiently and stably transfected cells. Its EC_{50} of 2 nM in stably transfected cells is the most potent to date of any dimerizer for the activation of gene transcription. To assess how weak a protein-ligand interaction is compatible with efficient transcription, the AP1889 (**1f**) half of **1i** was replaced with the drastically weaker monomer of **1h** (monomer IC_{50}: 1530 nM) to afford dimerizer **1j**. Surprisingly, **1j** exhibited good, though 7-fold weaker, activity in transiently transfected cells versus AP1903 (**1b**), and retained some activity in stable cell lines. **1j** also displayed a unique dose–response curve. Whereas increasing doses of other dimerizers induced a peak level of expression and then leveled off, **1j** quickly dropped to baseline after achieving its peak. This is most probably due to an excess of the AP1903 subunit of **1j** serving as a self-antagonist through the accumulation of monomeric (rather than dimeric) complexes.

To further probe the role of binding affinity, and to reduce the number of chiral centers and overall molecular weight, we replaced the 2-arylbutyric acid bottom portion of AP1903 with radically different structures. Of particular interest was dimerizer AP1966 (**1k**), in which the trimethoxyphenylbutyric acid bottom was replaced with a 3-pentyl urea. While urea-based ligands of wild-type FKBP had been reported in the literature,[13] due to the branched nature of the 3-pentyl group, the monomer of **1k** exhibited far greater selectivity for F36V-FKBP than wt-FKBP (64 nM vs 14 μM). The resulting dimerizer exhibited an EC_{50} only 2–3-fold reduced from that of AP1889 (**1f**) in both transient and stable assays, despite its radically simplified structure, and peak expression levels were also greater than those for AP1889 in both settings.

Conclusion

AP1903 (**1b**), a dimerizer with engineered specificity and tight binding affinity for the designed FKBP mutant F36V, potently activates Fas-mediated apoptosis and other signalling processes in appropriately engineered cells.[9,11] However the molecule is surprisingly inefficient at activating transcription, only functioning in transiently transfected cells where F36V-transcription factor fusion proteins are present at high levels. The analysis presented here indicates that while dimerizer potency in signalling appears largely driven by binding affinity, the structure activity relationship for transcription is more subtle, with kinetic and/or geometric factors also contributing. Indeed, we found that *reducing* the binding affinity of AP1903 dramatically enhanced activity, suggesting that tight binding prevents the efficient and dynamic assembly of functional dimerized complexes. These insights led to the design of novel, simpler dimerizers such as AP1889 (**1f**) and AP1966 (**1k**) that potently activate targeted gene expression in both transiently and stably transfected cells expressing F36V-FKBP fusion proteins. These homodimeric ligands, engineered to bind minimally to endogenous FKBPs, offer an alternative approach to the rapamycin-based heterodimerizer system[3,6] for inducing gene expression in stable cell lines and transgenic animals.

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