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Anthraquinone-peptides as Inhibitors of AP-1 Transcription Factor

Taeeba Ijaz, Phoung Tran, Ketan C. Ruparelia, Paul H. Teesdale-Spittle,† Samantha Orr and Laurence H. Patterson*

School of Pharmacy & Pharmaceutical Sciences, De Montfort University, Leicester, LE1 9BH, UK

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Abstract—Peptide-1-[*N*-{2-succinamidylethyl}amino]anthraquinones containing five–seven amino acid residues including the KCR motif important in AP-1 protein binding to DNA have been synthesised as potential transcription factor inhibitors. These anthraquinone-peptides showed DNA intercalative binding and inhibition of AP-1 protein binding to its DNA consensus sequence.

The transcription factor AP-1 which include the fos and jun bZIP protein families, plays an important role in both cell proliferation and malignant transformation.[1] AP-1 proteins have a highly conserved sequence motif lysine-cysteine-arginine (KCR) in the DNA binding domain.[2] Binding of the cfos-cjun heterodimer to its DNA binding domain is in part regulated by a redox mechanism through reversible conversion of the cysteine residue to its oxidation products.[2] Whilst others have explored the application of partially truncated AP-1-like peptides[3] these have been too long to be of value in designing potential chemotherapeutic agents. It was considered that certain truncated peptides would have the potential to bind the DNA AP-1 consensus sequence under the same conditions as the native AP-1 protein. Incorporation of an intercalating agent was anticipated to facilitate initial DNA binding and nuclear accumulation of the resulting conjugate peptides. In this paper the synthesis and DNA binding of potential inhibitors of AP-1 transcription factor binding and with intrinsic drug-like properties is described.

Truncated AP-1-like peptides, of five–seven residues, bearing the KCR motif were attached at the N-terminus to an intercalating anthraquinone moiety **2** through an amino acid type linker. We rationalised that the non-selective binding of the intercalating moiety should also stabilise the weaker specific binding of the peptide, compared to the extended, dimeric native protein. The linker, which in addition to ensuring minimal disruption of the AP-1 binding domain by DNA interaction of the intercalating moiety, was also required to facilitate a spatial arrangement which would allow the unrestricted movement of the peptide with respect to the intercalated chromophore.

Figure 1 shows that the synthesis of **2** involved preparation of the intercalator-linker moiety by amination of 1-chloroanthraquinone with ethylenediamine to afford **1**.[4]

This was subsequently reacted with succinic anhydride, which underwent ring opening to yield 1-[*N*-{2-succinamidylethyl}amino]anthraquinone **2**.[5] Eight Fmoc protected, resin bound peptides were synthesised commercially and subsequently acylated with **2**.[6] Resin-supported peptides facilitated the use of excess acylating components in the conjugate synthesis.

Binding to DNA and displacement of AP-1 protein of all eight peptide conjugates and their respective free peptides are shown in Table 1. DNA binding was measured by an increase the DNA melting temperature (helix to coil conversion) and was generally higher with peptides containing the most basic amino acid residues. For example **2**-AKCRNRA conjugate has a ΔT_m 4 °C higher than the **2**-AAKCRAA. The intercalative nature of the DNA binding of the conjugates was supported by their ability to displace ethidium bromide, the archetypical

*Corresponding author at present address: The School of Pharmacy, 29–39 Brunswick Square, London WC1N 1AX. Tel.: +44-(0)-207-753-5883; fax: +44-(0)-207-753-5964; e-mail: medchem@cub.ulsop.ac.uk

†Present address: School of Biological Sciences, Victoria University of Wellington, Kirk Building, Kelburn Parade, PO Box 600, Wellington, New Zealand.

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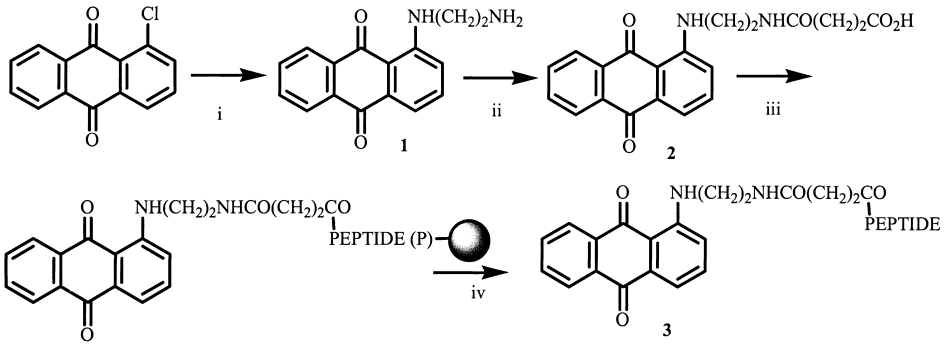

Figure 1. Synthesis of anthraquinone-peptide conjugates. Conditions: (i) Ethylenediamine, 25 °C, 72 h; (ii) succinic anhydride, DMF, 25 °C, 3 h; (iii) PyBOP, DIEA, DMF, 25 °C, 3 h; (iv) EDT (5%), H_2O (5%), TIS (5%)/TFA; P = Protected. Peptide = ARCKA; AKCRA; AKSRA; AKCRNA; AKCRKA; AKCRNRA; AKCRKRA; AAKCRAA.

DNA intercalating agent.[7] The ethidium displacement is almost certainly a result of competition for the intercalative binding site for which the conjugate **2**-AKCRKRA were more avidly bound than **2**-AKCRNA. This is consistent with differences between these two compounds in their effects on DNA melting temperature.

Having demonstrated DNA binding of the peptide-anthraquinone conjugates, these compounds were investigated for their ability to displace AP-1 protein binding from its DNA consensus sequence using the electrophoretic mobility shift assay (EMSA). The respective free peptides were also assayed for comparison. The assays were conducted using MDA-468 cell nuclear fraction shown to contain AP-1 family binding proteins.[8] The EMSA was performed in the presence of the reducing agent dithiothreitol (DTT). This was because the KCR motif was incorporated in all (but one) of the peptides prepared and hence DTT was used to ensure that the peptides were maintained in a reduced state most favourable to DNA binding. Preliminary experiments demonstrated that the cjun cfos heterodimers did not bind to the AP-1 consensus sequence if the EMSA was performed in the absence of DTT. This supports previous evidence that the affinity of the AP-1 binding proteins for its DNA cognate sequences is dependent on the reduced state of the specific cysteine residue of the highly conserved KCR motif, found in the DNA binding domains of this family of proteins.[2] Figure 1 shows a typical EMSA gel for **2**-AKCRKRA and demonstrates that displacement of AP-1 from its DNA consensus sequence is dependent on the concentration of the peptide conjugate. A concentration dependent AP-1 displacement was shown for all eight peptide-conjugates. The results in Table 1 show that all the peptides-conjugates displaced AP-1 much more effectively (i.e. have lower band intensities on EMSA autoradiographs) when compared to their respective free peptides. The results also show that the peptide conjugates containing the more basic sequences **2**-AKCRNA, **2**-AKCRKA, **2**-AKCRKRA and **2**-AKCRNRA were the most effective in displacing the AP-1 protein from its DNA consensus sequence. In summary, the whole assembly provides a combination of the high DNA affinity of an intercalator coupled with an AP-1 displacing peptide. Whether reversible oxidation of the KCR motif containing conjugates will modulate their DNA binding and hence inhibitory activity requires investigation (Fig. 2).

Table 1. Interactions with DNA and displacement of AP-1 binding

Peptide sequence	Free peptide[a]	Peptide-anthraquinone conjugate[a]			
	×1[b]	×0.1[b]	×1[b]	ΔT_m °C[c]	EtBr % quench[d]
X-ARCKA	98	79	51	4.8	—
X-AKCRA	99	78	43	7.5	—
X-AKSRA	99	73	41	5.5	—
X-AKCRNA	82	67	32	5.5	5
X-AKCRKA	83	68	39	7.3	—
X-AKCRNRA	87	70	38	10.3	—
X-AKCRKRA	86	61	26	9.0	45
X-AAKCRAA	93	79	56	6.0	—

[a]Free peptide: X = H; conjugate; X = 1-[*N*-{2-succinamidylethyl}amino]anthraquinone **2**; A = Ala; K = Lys; C = Cys; N = asparagine; R = Arg; S = Ser.

[b]EMSA gel band intensities (% control, mean of duplicates) were quantitated densitometrically; ×1 = 16.8 μM free peptide or peptide conjugate; ×0.1 = 1.68 μM peptide conjugate.[8]

[c]ΔT_m is the difference between the melting temperature of calf thymus DNA in the presence and absence of **2**-peptide conjugate at a 10:1 ratio of DNA/drug[9]; T_m of calf thymus DNA was 71.0 °C.

[d]% maximum loss of ethidium bromide (EtBr) fluorescence bound to DNA[9] (ratio 1:3) in presence of **2**-peptide conjugate. — = not determined.

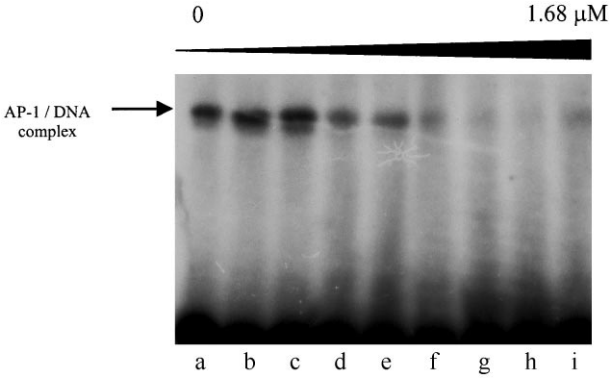

Figure 2. Autoradiograph of an EMSA gel showing **2**-peptide conjugate displacement of AP-1 protein from its ^{32}P-DNA (2.1 nM) consensus sequence. Lane a: control, b: 0.21 μM, c: 0.42 μM, d: 0.63 μM, e: 0.84 μM, f: 1.05 μM, g: 1.26 μM, h: 1.47 μM, i: 1.68 μM **2**-AKCRKRA.

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4. 1-[*N*-{2-Aminoethyl}amino]anthraquinone, **1**. 1-Chloroanthraquinone (1.0 mmol) was dissolved in 80-fold excess ethylenediamine, stirred at rt for 72 h, and diluted with water. The red precipitate was eluted from a flash silica chromatography column with CH_2Cl_2/increasing CH_3OH (0–50%) gradient (yield 36%), mp 148 °C: ν_{max}/cm^{-1} 3350 (NH_2), 2590 (CH), 1690 (C=O), 1600 (C=C), 1280 (NH), 700 (ArCH); δ_H (CD_3OD): 3.0 (m, 2H; C*H*$_2$NH$_2$), 3.5 (t, 2H; NHC*H*$_2$), 7.25 (d, 1H; Ar*H*), 7.5 (m, 2H; Ar*H*), 7.8 (m, 2H; Ar*H*), 8.15 (d, 2H; Ar*H*), 8.3 (d, 1H; Ar*H*); δ_C (CD_3OD): 41.86, 43.25, 116.67, 119.31, 127.0, 134.29, 135.01, 136.55, 144.5, 181.5, 183.27; *m*/*z* $(M+H)^+$ 267; CHN: Found C (68.99%), H (5.33%), N (9.82%). Calculated with 0.75 H_2O, C (68.69%), H (5.55%), N (10.02%).
5. 1-[*N*-{2-Succinamidylethyl}amino]anthraquinone **2**. Compound **1** (0.37 mmol) and succinic anhydride (0.45 mmol) in DMF were stirred under N_2 for 3 h at rt, diluted with water and the product was extracted with CH_2Cl_2, dried over $MgSO_4$ and reduced in vacuo. The red product (72% yield) was chromatographed as described above.[4] Mp 156 °C: ν_{max}/cm^{-1} 3400 (COOH), 3300 (NH), 1710 (C=O), 1680 (NHC=O), 1630 (C=C), 1280 (NH), 700 (ArCH); δ_H (DMSO): 2.4 (m, 4H; NHC*H*$_2$C*H*$_2$NH$_2$), 3.2 (t, 2H; COC*H*$_2$), 3.4 (t, 2H; C*H*$_2$COOH), 7.3 (d, 1H; Ar*H*), 7.4 (d, 1H; Ar*H*), 7.8 (m, 2H; Ar*H*), 8.1 (m, 2H; Ar*H*), 9.7 (t, 1H; Ar*H*); δ_C (DMSO): 40.25, 113.0, 115.29, 126.38, 126.54, 132.46, 134.14, 134.5, 134.63, 135.71, 151.01, 151.67, 183.01, 184.0; *m*/*z* $(M+H)^+$ 367; CHN: Found C (64.93%), H (5.04%), N (7.56%). Calculated with 0.75 H_2O, C (64.78%), H (4.99%), N (7.56%).
6. The intercalator-linker, **2** (4 equiv) was added to the peptide bound resin (in anhydrous DMF) followed by PyBOP (4 equiv) and DIEA (8 equiv) at room temperature and kept under N_2 for 3 h followed by sequential washing with DMF, CH_3OH, $(C_2H_5)_2O$. Deprotection and cleavage from resin used EDT (5%), TIS (5%) and water (5%) in TFA for 5 h at rt. The product was filtered, washed with TFA, reduced in vacuo at rt, triturated with cold anhydrous $(C_2H_5)_2O$ and dried over P_2O_5.
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8. Xanthoudakis, S.; Curran, T. In *Methods in Enzymology*; Colowick, S. P., Kaplan, N. O., Eds.; Academic Press: New York, 1994; Vol. 234, pp 163–174. In these studies AP-1 protein (approx 0.2 μg/mL Fos and Jun protein) containing cell nuclei extract (0.18 mg/mL) was isolated from MDA-468 breast cancer cells. EMSA used AP-1 protein (20 ng), ^{32}P- end labelled 5′-CGCTTGATGAGTCAGCCGGAA-3′ duplex (2.1 nM), 2-peptide conjugate:DNA ratio 200–8000:1) or free peptide:DNA ratio 2000–8000:1).
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