ANTHRAQUINONE DYES: A NEW CLASS OF POTENT INHIBITORS OF MITOCHONDRIAL ADENINE NUCLEOTIDE TRANSLOCATION AND OXIDATIVE PHOSPHORYLATION

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1. Introduction

During the last two decades triazinyl textile dyes have been extensively used as ligands for affinity chromatography for the purification of enzymes and other proteins. In addition to their usefulness as ligands the dyes turned out to be strong inhibitors for various nucleotide-dependent enzymes [1].

The carrier-catalyzed mitochondrial adenine nucleotide transport is known to be specifically inhibited by the atractylates and bongrekate [2–4]. This communication describes the effect of a series of anthraquinone derivatives on the binding, transport and subsequent oxidative phosphorylation of ADP in rat liver mitochondria and submitochondrial partciles. A systematic study led to a very defined structure–inhibitor activity relationship of anthraquinones with respect to the mitochondrial adenine nucleotide transport system. The minimum structure for effective inhibition of ADP transport is shown to be represented by 1-amino (or -hydroxy)-anthraquinone-2 or 3-sulfonic acid. Some results of this work have been presented in part as a poster contribution [5].

2. Materials and methods

1-Amino-anthraquinone (I), anthraquinone-2-sulfonic acid (II), 1,4-diaminoanthraquinone (III), 1-amino-4-hydroxy-anthraquinone (IV), 1,4-dihydroxy-anthraquinone (V), 1,2-dihydroxy-anthraquinone (VI), anthraquinone-1,5-disulfonic acid (VII), anthraquinone-2,6-disulfonic acid (VIII), 1-amino-2-methoxy-4-hydroxy-anthraquinone (IX), 1,6-diamino-4,8-dihydroxy-anthraquinone-3,7-disulfonic acid (acid blue 45; CI 63010) (X), 3,4-dihydroxyanthraquinone-2-sulfonic acid (mordant red 3; CI 58005) (XII), 1-amino-anthraquinone-2-sulfonic acid (XIV), acid blue 25 (CI 62055) (XVI), acid blue 40 (CI 62125) (XVII), acid blue 41 (CI 62130) (XVIII) and reactive blue 2 (CI 61211) (XXII) were purchased from EGA Chemie. Acid blue 55 (CI 63315) (XV), reactive blue 19 (CI 61200) (XX) and reactive blue 29 (XXIII) were generous gifts of Bayer AG, Leverkusen. Uniblue A (XIX) was from Eastman and reactive blue 4 (XXI) from Serva Chemie. Atractyloside and carboxyatractyloside were obtained from Boehringer. Atractyligenin was a generous gift of Professor Mayer, University of Braunschweig, bongrekate a gift of Professor Berends, University of Delft. [^{14}C]ADP and 2′d-[^{14}C]ATP were products of NEN chemicals. rro-[^{14}C]ADP was prepared as in [6]. 2′d-[^{14}C]ATP was converted to the corresponding diphosphate as in [7].

Abbreviations: Atrac, actractyloside; BKA, bongrekate; CAT, carboxyatractyloside, CI, colour index constitution number; 2′d ADP, 9-(β-D-2′-deoxyribofuranosyl)adenine-5′-diphosphate; rro ADP, 2,2′[1-(9-adenyl)-1′-diphosphoryl-oxymethyl]-dihydroxydiethylether; Ap_5A, P^1, P^5-di(adenosine-5′-)pentaphosphate; TLC, thin-layer chromatography; SMP, submitochondrial particles

2.1. *Syntheses and purification of dyes*

1,4-Dihydroxy-anthraquinone-2-sulfonic acid (XI) was prepared according to [8] and 1-amino-4-bromo-anthraquinone-2-sulfonic acid (XIII) according to [9]. Separation of the reaction products was done by DEAE-52 cellulose column chromatography using a linear gradient of 0–1.0 M triethylammonium bicarbonate (pH 7.5). Purity was controlled spectrophotometrically and by TLC on PEI-cellulose plates (F 1440, Schleicher and Schüll) using 1 M triethylammonium bicarbonate (pH 7.5) as mobile phase. All the other

investigated dyes were purified by preparative TLC on activated silica gel plates using tetrahydrofuran:H_2O, 48:7 (v/v) as mobile phase [10]. Halogenated dyes (XXI–XXIII) were desactivated by heating 20 mg dye in 1 ml 0.01 N NaOH for 4 days at 60°C whereby the course of the reaction was followed by TLC. Every 24 h 0.1 ml 0.01 N NaOH was added and the reaction mixture finally neutralized with 1 N HCl.

2.2. *Binding and translocation measurements*

Mitochondria were prepared from rat liver (male Wistar rats Bor: WISW, SPF TNO: 150–200 g) according to [11]. Protein was determined by the biuret method. Mitochondria (2.5 mg protein) were incubated at 5°C in a medium of 70 mM sucrose, 210 mM mannitol, 1 mM triethanolamine, 40 μM Ap_5A, and oligomycin (4 μg/mg of protein), pH 7.2, in a total volume of 290 μl. Separation of mitochondria from the incubation mixture (50 μl aliquots) was done by centrifugation through a silicone oil layer (AR-200, Wacker Chemie) in microvials using a Beckman Microfuge. The radioactivity in the denatured (15% $HClO_4$) sediment was measured with a liquid scintillation counter using Instagel II (Packard). Differentiation between carrier-linked, i.e., inhibitor-sensitive (specific), and noncarrier-linked, i.e., inhibitor-insensitive (unspecific), binding as well as exchange (uptake) with the endogenous adenine nucleotide pool was performed in analogy to [12].

2.3. *Oxidative phosphorylation*

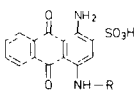
Rat liver submitochondrial particles were prepared according to [13] and oxidative phosphorylation was followed via ^{32}P-incorporation into ATP. Buffer A: 250 mM sucrose, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris, 40 μM Ap_5A, 10 mM succinate and 2.5 mM P_i (pH 7.4). Quantitative TLC-analysis of [^{32}P]ATP in the denatured (15% $HClO_4$) incubation mixture was performed as in [14].

3. Results

The structures of some of the dyes investigated are shown in table 1.

The results in table 2 clearly demonstrate that one can differentiate in analogy to [12] between dye-sensitive (carrier-specific) and dye-insensitive (carrier-unspecific) binding of [^{14}C]ADP. Preincubation of mitochondria (expt. 6,8) with compounds (XV, XIX–XXIII) yields the same amount (0.20 nmol [^{14}C]ADP/mg protein) of carrier-unspecific bound [^{14}C[ADP compared to that found with the known inhibitors (expt. 5,7). Subtraction of the values of expt. 3 and 2, respectively, from that of expt. 1 reveals, on the other hand, a significantly higher value for dye-sensitive bound [^{14}C]ADP compared to atractylate-sensitive. Addition of dye to the incubation medium after carrier-specific bound [^{14}C]ADP has been replaced by atractylates (expt. 4) yields the same value for inhibitor-sensitive bound [^{14}C]ADP as obtained with dye alone. It is thus evident that the dyes, i.e., XV, XIX–XXIII, can displace a higher amount of presumably carrier-specific bound [^{14}C]ADP.

Table 1
Some structures of investigated anthraquinone dyes



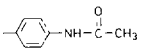
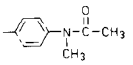
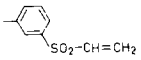
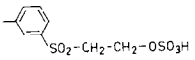
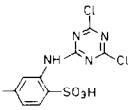
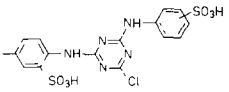
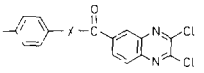
XVI	R:	
XVII	R:	
XVIII	R:	
XIX	R:	
XX	R:	
XXI	R:	
XXII	R:	
XXIII	R: partial structure; patent Bayer AG	

Table 2
Inhibitor-sensitive and -insensitive binding of [^{14}C]ADP to rat liver mitochondria

Expt.	Sequence of additions	[^{14}C]ADP (nmol/mg of protein)
1	[^{14}C]ADP–1 min	4.10 ± 0.10[a]
2	[^{14}C]ADP–1 min –Atractylates–1 min	3.30 ± 0.12
3	[^{14}C]ADP–1 min –Dye–1 min	2.65 ± 0.25
4	[^{14}C]ADP–1 min –Atractylates–1 min –Dye–1 min	2.70 ± 0.26
5	Atractylates–1 min –[^{14}C]ADP–1 min	0.21 ± 0.03
6	Dye–1 min –[^{14}C]ADP–1 min	0.20 + 0.02
7	Atractylates–1 min –[^{14}C]ADP–1 min –Dye–1 min	0.19 ± 0.02
8	Dye–1 min –[^{14}C]ADP–1 min –Atractylates–1 min	0 20 ± 0.02

[a] Values reported are taken from four independent experiments, range of confidence is 95%

Incubation of mitochondria was done as in section 2 and terminated by centrifugation through a silicone oil layer. [^{14}C]-ADP, 60 μM; Atractylates (Atrac, Atractyligenin, CAT) and dye (XV, XIX–XXIII) 500 μM

In this context, dye-sensitive and -insensitive binding of ADP analogues which were shown to be specifically bound but not transported by the carrier protein was investigated (table 3). The results of expt. 2,4 as well as 5 and 7 indicate that a fixed amount of atractylate-insensitive bound ADP analogues, i.e., ~0.1 nmol 2′d ADP and 0.46 nmol rro ADP, can be additionally displaced by the investigated dyes.

Exclusion of any transport activity also was achieved by preincubation of mitochondria with bongrekate which is known to inhibit mitochondrial ADP uptake in contrast to the atractylates by binding to the carrier protein from the inside. The results (cf. table 4) indicate that a higher amount of [^{14}C]ADP is displaced by Atrac or CAT than by the dyes, whereas inhibitor-insensitive binding is almost unchanged.

Inhibition studies during the linear phase [15] of mitochondrial [^{14}C]ADP (60 μM) uptake revealed that compounds I–X showed no effect up to 500 μM. [^{14}C]ADP uptake is reduced to 0.61 (nmol/mg protein), i.e., 70% when the dyes XI–XIV and XVI–XVIII (250 μM) are added simultaneously. The derivatives XV and XIX XXIII, however, turned out to inhibit [^{14}C]ADP uptake completely.

As shown in table 5 two different types of inhibition were found for [^{14}C]ADP uptake in the presence of dyes. The graphical determination (Dixon-plot) of

Table 3
Inhibitor-sensitive and -insensitive binding of [^{14}C]ADP analogues to rat liver mitochondria

Expt.	Sequence of additions	[^{14}C]2′d ADP (nmol/mg protein)	[^{14}C]rro ADP (nmol/mg protein)
1	ADP analogue–1 min	0 83 ± 0.04[a]	2.01 ± 0.09
2	ADP analogue–1 min –Atractylates–1 min	0.30 ± 0.03	1.75 ± 0.06
3	ADP analogue–1 min –Dye–1 min	0.20 ± 0.02	1.31 ± 0.06
4	ADP analogue–1 min –Atractylates–1 min–Dye–1 min	0 21 ± 0.02	1.28 ± 0.07
5	Atractylates–1 min –ADP analogue–1 min	0.32 ± 0.03	1.77 ± 0.04
6	Dye–1 min–ADP analogue –1 min	0.19 ± 0.02	1.29 ± 0.04
7	Atractylates–1 min –ADP analogue–1 min–Dye–1 min	0.20 ± 0.02	1.30 ± 0.05

[a] Values reported are taken from 4 independent experiments; range of confidence is 95%

Incubation of mitochondria was done as in section 2 and terminated by centrifugation through a silicone oil layer. 2′d[^{14}C]ADP, 70 μM; rro[^{14}C]ADP 200 μM, Atractylates (Atrac, Atractyligenin, CAT) and dye (XV, XIX–XXIII) 500 μM

Table 4
Inhibitor-sensitive and -insensitive binding of [^{14}C]ADP to bongrekate (BKA)-treated rat liver mitochondria

Sequence of additions	[^{14}C]ADP (nmol/mg protein)
BKA–[^{14}C]ADP–1 min	1.10 ± 0.09[a]
BKA–[^{14}C]ADP–1 min –Atrac or CAT–1 min	0.33 ± 0.05
BKA–[^{14}C]ADP–1 min –Dye–1 min	0.52 ± 0.07
BKA–Atrac or CAT–1 min –[^{14}C]ADP–1 min	0 14 ± 0.04
BKA–Dye–1 min –[^{14}C]ADP–1 min	0.21 ± 0.04

[a] Values reported are taken from 4 independent experiments; range of confidence is 95%

Mitochondria (2.5 mg protein) were preincubated for 10 min at 24°C in a medium of 70 mM sucrose, 210 mM mannitol, 1 mM triethanolamine, 40 μM Ap_5A and 500 μM BKA, pH 7.2 (total vol. 290 μl) and cooled to 5°C. Incubation was terminated at the indicated time by centrifugation through a silicone oil layer. [^{14}C]ADP, 60 μM; Atrac, CAT and dye (XV, XIX–XXIII) 500 μM

the inhibitor constants of compounds XV, XXII, XXIII revealed a non-competitive type. V_{max}, however, is not significantly decreased nor is K_m changed. The inhibitory action of these dyes thus seems to be a mixed type, i.e., partially competitive–non-competitive.

The inhibitory action of the investigated dyes on mitochondrial ADP uptake is further demonstrated by their effect on ADP-stimulated respiration which is abolished by addition of dye prior to ADP (not shown) as well as on oxidative phosphorylation of externally added ADP (cf. table 6). Moreover, compound XV turns out as a potent inhibitor of oxidative phosphorylation of ADP in submitochondrial particles.

Table 5
Inhibition of mitochondrial [^{14}C]ADP uptake by anthraquinone dyes

Dye	K_i (μM)	$K_{m'}$ (μM)	V_{max} (nmol . (min × mg)$^{-1}$)	Type
XIV[a]	25	200	14	Competitive
XV[b]	40	100	19	'Mixed'
XXII[b]	15	89	11	'Mixed'
XXIII[b]	20	86	13	'Mixed'

[a] Values given were derived from a Lineweaver-Burk plot; XIV, 100 μM; K_m (ADP) 40 μM

[b] K_i values were calculated from Dixon-plots, $K_{m'}$ and V_{max} from replots of m vs $1/S$, respectively

Sampling for rate measurements in the presence of dyes was performed within the first 4–15 s of [^{14}C]ADP uptake as in [15]

Table 6
Effect of dyes on oxidative phosphorylation of ADP in mitochondria and submitochondrial particles

	[^{32}P]ATP (nmol/mg protein)
Mitochondria:	
Control (10 min)	344
+ Atrac	72
+ Dye (XV)	78
+ Dye (XXIII)	26
SMP:	
Control (10 min)	5.7
+ Atrac	2.5
+ Dye (XV)	0.7

Mitochondria (0.5 mg protein) and SMP (0.6 mg protein) were incubated in 235 μl buffer A at 5°C. Final conc.: $^{32}P_i$, 2 mM; ADP, 650 μM; preincubation (1 min) with Atrac 80 μM or dye 100 μM.

4. Discussion

This in vitro study introduces a new class of potent inhibitors of mitochondrial adenine nucleotide translocation and oxidative phosphorylation. The investigated substances are commonly used as synthetic dyes and exhibit two remarkable features when compared to the known inhibitors Atrac, CAT and BKA, namely colour and covalent binding properties. Besides their chemical nature the dyes differ from the known inhibitors in their properties towards the adenine nucleotide carrier-system.

The amount of displaced ADP is significantly higher compared to that found with the atractylates. Furthermore, the displacement of substrate analogues which are carrier-specific bound but not transported by the carrier protein [15] shows that the dyes can displace a certain amount of carrier-specific but atractylate-insensitive bound ADP analogues. With BKA-treated mitochondria, on the other hand, more ADP is displaced by the atractylates than by the dyes.

The dyes inhibit mitochondrial [^{14}C]ADP uptake with inhibition constants of 15–40 μM in a partially competitive–non-competitive manner, except com-

pound XIV which acts competitively. The natural product Atrac is known as a competitive ($K_i \sim 10^{-7}$ M) and CAT as a non-competive inhibitor ($K_i \sim 10^{-9}$ M) [2,3]. The results suggest that the accessibility for the dyes to the substrate binding site at the carrier protein is different to that for the atractylates. The dyes thus might act on a different conformational state of the carrier–ADP complex. This becomes more evident by comparison of the chemical structures and functional groups of the various inhibitors. The active part of Atrac was shown to be the genin moiety, i.e., atractyligenin with an essential carboxylic group at the C(4)-atom [2]. CAT bears an additional carboxylic group at that position. Also in this study a substructure could be identified as features common to all inhibiting dyes. This minimum structure for effective inhibition is represented by the chromophore 9,10-dihydro-9,10-dioxo-anthracene with two essential substituents, i.e., a C(1)-positioned hydroxy- or amino group and a C(2)- or C(3)-positioned sulfonic group. An additional ionic substituent at the C(6) position impedes any inhibitory action (cf. compound X). Furthermore, it is most likely that for its active form the chromophore has to build up intramolecular hydrogen-bridges between its carbonyl functions and the C(1)- and C(4)-positioned H-donating groups.

The imino-bridged residue in compounds XVI–XXIII seems to be of minor importance, although it might be expected that the halogenated dyes (XXI–XXIII) as well as compounds XIX and XX inhibit ADP uptake due to covalent bond formation with the carrier protein. In this case deviation from competitive inhibition could occur. The findings with compound XV as well as the experimental conditions exclude this assumption, although the type of inhibition for the desactivated dyes (XXI–XXIII) which, on the other hand, are still potent inhibitors (not shown) has not been determined. Experiments on the covalent labelling of the carrier protein with reactive dyes as well as the synthesis of radioactive inhibitors are in progress.

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