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Localization of a Nonintercalative DNA Binding Antitumour Drug in Mitochondria: Relationship to Multidrug Resistance*

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Abstract—*The bis-(*n*-butyl) quaternary salt of* N,N′*-bis-(6-quinolyl)terephthalamide (QBQ), a fluorescent antitumour compound in the phthalanilide series which is thought to bind to the minor groove of the DNA double helix, has been investigated with respect to its* in vitro *activity and subcellular localization. Cultured MCF-7 human breast carcinoma cells concentrated QBQ in mitochondria by a time-dependent process which was inhibited by the ionophore valinomycin, suggesting a possible mode of antitumour action of QBQ through mitochondrial poisoning. Growth of cultured P388 murine leukaemia cells was inhibited* 50% *in the presence of* 0.52 μM *QBQ, and multidrug-resistant P388 sublines developed for resistance to actinomycin D, vincristine, Adriamycin® and the phthalanilide NSC* 38280 *were cross-resistant to the drug. Cross-resistance was reduced in all lines by the presence of* 11 μM *verapamil, suggesting that a transport resistance mechanism operates on QBQ. The actinomcyin D-resistant P388 cell line was found to be cross-resistant to the aromatic cations rhodamine* 123, *which binds to proteins, and ethidium and pyronin Y, which bind intercalatively to DNA. Thus mitochondrion-specific drugs with different macromolecular binding properties all appear to be excluded by multidrug-resistant cells.*

INTRODUCTION

THE TERM 'phthalanilides' has been applied to the group of bis-charged aromatic compounds, generally derivatives of terephthalic acid [1], which were originally designed as inhibitors of phospholipid metabolism [2] and were later found to bind to the minor groove of double-stranded DNA [3, 4]. Clinical trials were conducted on several derivatives but toxicity masked any potential activity. Cain and co-workers developed the phthalanilides by producing bisquaternary salt derivatives. By varying the length of the alkyl group, the lipophilic–hydrophilic balance could be varied and thus antitumour activity optimized [4, 5].

The phthalanilides are members of a larger class of positively charged agents which binds strongly and non-intercalatively to DNA, probably in the minor groove [3, 6, 7]. Although a wide variety of biological effects including antitumour, antiparasitic, antiviral and antibacterial activity have been reported [6, 7], the mode of action of these compounds is not yet fully understood. In this study, we have selected a quaternized bis-quinolinium phthanilide derivative (QBQ: see structure in Fig. 1) which is fluorescent, thereby allowing visualization of its intracellular location. QBQ was selected from others in the homologous series because of its antitumour activity against murine L1210 [5]. A human breast carcinoma line (MCF-7) [8] has been used to study mitochondrial localization. Comparisons have been made with rhodamine 123, a fluorescent aromatic cationic compound which is selectively concentrated by mitochondria of mammalian cells [9] and which shows increased cytotoxicity towards some types of carcinoma cells [10], as well as with a number of other fluorescent compounds.

In order to understand better the basis of selective toxicity of QBQ and rhodamine 123, growth inhibition assays have been performed using a range of sublines of the murine P388 lymphocytic leukaemia, comprising lines which have been developed for resistance to vincristine [11], actinomycin D [12], doxorubicin [13], the phthalanilide derivative 2-chloro-4′,4″-di(2-imidazolin-2-yl)terephthalanilide (NSC 38280) [11] and amsacrine [14]. The results show that multidrug-resistant cells which have a

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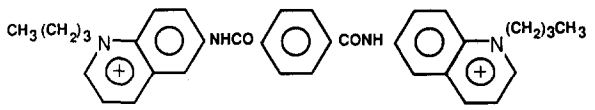

Fig. 1. Structure of QBQ. The counterion is p-methylbenzenesulphonate.

verapamil-sensitive drug resistance mechanism are cross-resistant to a range of mitochondrion-specific drugs with different protein and nucleic acid binding properties. The uptake of aromatic cations into mitochondria is discussed in relationship to mechanisms of energy-dependent efflux.

MATERIALS AND METHODS

Materials

QBQ was synthesized in the Cancer Research Laboratory [5], verapamil was from Knoll AG, F.R.G., and other compounds were from the Sigma Chemical Company.

Cell lines

Cultured MCF-7 cells [8] were provided by Dr G.J. Finlay. P388 cells were obtained in 1977 from the Developmental Therapeutics Program, National Cancer Institute, U.S.A. P/VCR, P/ACTD, P/NSC and P/ADR cells were obtained from Mason Research, Worcester, U.S.A. P/AMSA cells, originating from Dr R.K. Johnson [14], were obtained from Dr R.C. Jackson, Parke-Davis Division, Warner-Lambert Company, Michigan, U.S.A. P388 and its resistant sublines were initially passaged intraperitoneally in DBA/2J mice, and adapted to culture over a period of approximately 3 weeks.

Cell culture

MCF-7 cells were cultured in 96-well dishes in α-MEM with serum and antibiotics according to published methods [15]. P388 and its sublines were cultured in 24-well tissue culture dishes in a humidified atmosphere of 5% CO_2 in air at 37°C [16]. RPMI-1640 medium contained heat-inactivated foetal bovine serum (5%), 2-mercaptoethanol (50 μM), penicillin (10 units/ml) and streptomycin (100 μg/ml). Exponentially growing cultures (3×10^4 cells in 1 ml) were grown for 2 h before addition of drugs at 2-fold concentration increments. Cells were counted at the end of the incubation period (70 h) with an electronic cell counter. Assays were carried out in duplicate on two different occasions and the results averaged.

Observation of mitochondrial accumulation

MCF-7 cells were cultured on 12 mm circular glass microscope coverslips in 25 cm^2 dishes to allow subsequent mounting in a live cell observation chamber. This was constructed from a polysiloxical dental impression material (Coltene Inc., Altstätten, Switzerland) as 1 mm thick sheets, punched with a 10 mm hole and pressed on to a standard 25 × 75 mm microscope slide. Cells were observed in a Nikon Optiphot microscope equipped with a Model EF-D fluorescence attachment. Ultraviolet illumination (330–380 nm) was normally used but in some cases (rhodamine 123, ethidium and acridine orange) a 546 nm excitation filter and appropriate bandpass emission filter was used.

RESULTS

Subcellular localization of QBQ in MCF-7 cells

MCF-7 cells were grown in a culture dish on a microscope slide for several days to establish extensive logarithmic cell growth. Cells were then exposed to QBQ (2 μg/ml) for various times at 37°C before the coverslip was observed in a microscope culture chamber. After 1–2 h, a bluish diffuse cytoplasmic fluorescence was observed, and after 6–24 h blue particulate cytoplasmic structures were seen. Since QBQ at higher concentrations caused precipitation of crystals in the culture medium, the more soluble di-*n*-pentyl derivative [5] was used for some experiments (5 μg/ml), giving similar but more intensely fluorescent structures (Fig. 2). P388 cells also showed fluorescent particulate cytoplasmic structures, but the small volume of the cytoplasm made the identification of the structures difficult.

QBQ was next compared with rhodamine 123, a drug which is known to localize in mitochondria [9]. MCF-7 cells were incubated with rhodamine 123 (5 μg/ml) and when observed under ultraviolet or green excitation showed oval cytoplasmic structures which appeared to have a similar distribution but a different shape to those observed with QBQ. To determine the localization of QBQ more precisely, cells were incubated with a combination of the di-*n*-pentyl QBQ homologue (5 μg/ml) and rhodamine 123 (1 μg/ml) for 6 h and examined. Green particulate structures were observed with the fluorescence dominated by rhodamine 123 but with a shape similar to that observed with QBQ alone, indicating that QBQ homologue and rhodamine 123 both stain the same structures.

Cells were also stained with ethidium bromide pyronin Y and acridine orange (2 μg/ml, 2 h). Ethidium and pyronin Y stained mitochondria, whereas acridine orange intensely stained red–orange spherical structures. The low number and asymmetric cytoplasmic distribution of these structures, together with their size distribution (most structures were smaller than mitochondria but some were considerably larger) contrasted with that observed with QBQ, rhodamine 123, ethidium and pyronin Y.

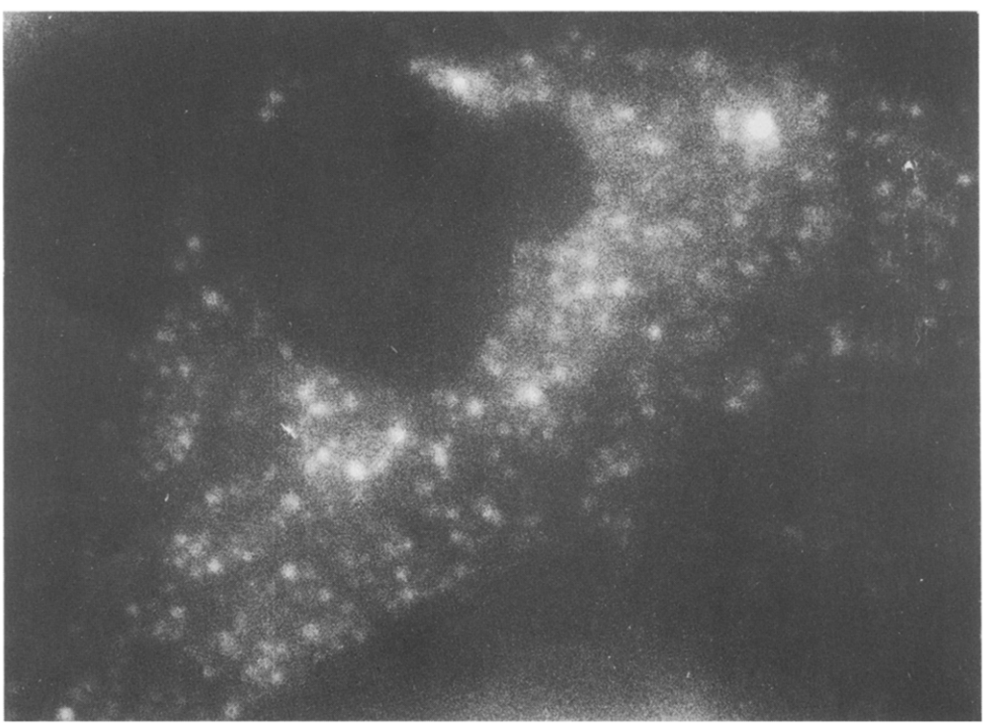

Fig. 2. *Appearance of MCF cells stained with the di-*n*-pentyl analogue of QBQ* (5 μg/ml; 24 h).

Valinomycin is known to block potently the uptake of rhodamines into mitochondria [17]. Cells were incubated with QBQ or its di-*n*-pentyl homologue in the presence of valinomycin (500 ng/ml), added either at the start of a 6 h incubation or 10 min before the end of the incubation. The distribution of bluish fluorescence became diffuse, although a small number of asymmetrically distributed particulate, spherical structures could also be seen. Valinomycin had a similar effect on the fluorescence of rhodamine 123.

Inhibition of cell growth

IC_{50} values, determined for QBQ and rhodamine 123 for a number of cell lines, are shown in Table 1. P388 was the most sensitive line (IC_{50} = 0.52 and 0.45 μM respectively) while P/ADR (resistant to Adriamycin®) and P/NSC (resistant to NSC 38280) were resistant to both drugs. The range of IC_{50} values was greater than 5-fold for QBQ (limited by the solubility of this compound) and greater than 500-fold for rhodamine 123. The IC_{50} value of QBQ for MCF-7 cells were measured under similar conditions and found to be 1.9 μM. IC_{50} values were also determined for ethidium bromide, pyronin Y, Adriamycin®, etoposide, actinomycin D and vincristine using the P388 and P/ACTD cell lines (Table 2). Cross-resistance was found for all drugs tested.

Effect of verapamil of growth inhibition

The addition of verapamil at a concentration (5 μg/ml) which was not itself cytotoxic, but nevertheless caused some growth inhibition (20–50%) when administered alone, induced a large increase in the sensitivity to rhodamine 123 and a smaller increase in sensitivity to QBQ (Table 1). The increases were greatest for the most resistant lines, with the range of IC_{50} values for rhodamine 123 being compressed from >500-fold to 25-fold.

Table 1. *Inhibition of cell lines by QBQ and rhodamine* 123

	IC_{50} value (μM)			
	QBQ		Rhodamine 123	
Cell line	−Ver	+Ver	−Ver	+Ver
P388	0.52	0.34	0.45	0.14
P/AMSA	0.88	0.74	1.2	0.57
P/ACTD	2.2	0.54	190	3.5
P/NSC	>2.2	0.54	>250	2.9
P/ADR	>2.2	0.16	>250	2.9
P/VCR	>2.2	0.83	170	2.7
LLTC	>2		34	
MCF-7	1.9		8.3	

Table 2. In vitro *growth inhibition of P388 and P/ACTD cells by mitochondrial stains and other compounds*

	IC_{50} value (nM)		
Drug	P388	P/ACTD	Cross-resistance
BQ	0.52	2.2	4.2
Rhodamine 123	0.45	190	420
Ethidium bromide	110	2300	21
Pyronin Y	17,000	79,000	4.6
Actinomycin D	0.12	1.4	12
Doxorubicin	12	950	79
Vincristine	0.36	21	58

DISCUSSION

The fluorescence of QBQ has allowed the demonstration that this compound, like rhodamine 123 [9], ethidium [18] and pyronin Y [19], is concentrated by mitochondria (Fig. 2). QBQ is a nonintercalative DNA binder [3, 6] while ethidium bromide and pyronin Y bind to DNA by intercalation and rhodamines interact with proteins [20]. Since nonintercalative DNA binders are of interest because of their sequence specificity of DNA binding [6, 7] mitochondrial localization is an important consideration in the design of compounds which might interact with nuclear DNA receptors. One problem in ascertaining the cellular localization of QBQ is that in solution its fluorescence is strongly quenched by double-stranded DNA, although not by proteins (data not shown). The observed fluorescence may therefore reflect protein-bound drug rather than total drug, and might underestimate drug bound at DNA sites in mitochondria or in the nucleus. Since highly lipophilic drugs would be expected to show high binding to hydrophobic sites on proteins, the superiority of di-*n*-pentyl QBQ over the di-*n*-butyl homologue as a fluorescent marker could result from its greater protein binding.

QBQ and related compounds in the phthalanilide series have pronounced antileukaemia activity [4, 5] whereas ethidium, rhodamine 123 and pyronin Y have little activity in such systems. It is possible that the DNA binding properties of QBQ distinguish it from these other drugs. Ditercalinium, a bis-intercalating drug [21], is an active antileukaemia drug showing long-term delayed toxicity reminiscent of the phthalanilides [1, 4]. It binds strongly to DNA and results in the loss of mitochondrial DNA over a period of 24 h [22]. It will be interesting to determine whether QBQ also induces the loss of mitochondrial DNA. QBQ and other phthalanilides induce 'petite' (respiration-deficient) mutants in the yeast *Saccharomyces cerevisiae*, strongly implicating mitochondria as a site of drug concentration [23]. Phthlanilides also affect

mitochondria of L1210 cells [24], suggesting accumulation. However, it is not yet clear whether these compounds exert their antitumour effects through mitochondria.

The range of IC_{50} values obtained for different P388 cell lines using both QBQ and rhodamine 123 (Table 1), the reduction in IC_{50} values induced by the presence of verapamil, and previous studies with rhodamine 123 [25] all support the hypothesis that a verapamil-sensitive drug efflux mechanism [26] mediates the resistance of cells to aromatic cationic drugs. It has been proposed that this involves the accumulation of drugs in cytoplasmic vesicles (or the pumping of drugs across the plasma membrane) by an ATP-driven permease incorporating P-glycoprotein [27, 28]. Other mechanisms, including the concentration of drug in acidic vesicles and binding to intravesicular proteins, may also operate [27]. Drugs concentrated in cytoplasmic vesicles may subsequently be eliminated by exocytosis [29].

Accumulation of aromatic cations in mitochondria, like the accumulation in cytoplasmic vesicles, may have multiple mechanisms. Uptake of rhodamine 123 has been postulated to be driven by the mitochondrial membrane potential [10]. 10-Nonylacridine orange has been postulated to accumulate by an energy-independent process through specific mitochondrial protein binding [30]. Uptake of positively charged acridines into yeast mitochondria has been proposed to occur by a process which is sensitive to verapamil [31], as well as to a number of other drugs which overcome multidrug resistance in mammalian cells (B.C. Baguley, P.M. Turner and L.R. Ferguson, manuscript in preparation). Which of these mechanisms applies to the uptake of QBQ is not known. The valinomycin sensitivity of QBQ uptake suggests that an energy-dependent process is operating, perhaps driven by a mitochondrial membrane potential. However, it is by no means clear what the magnitude of the *in situ* mitochondrial membrane potential is [32], and whether it is sufficient for effective concentration of drug. Valinomycin could also act on other mechanisms by reducing mitochondrial ATP content. Further work is required to determine whether the susceptibility to multidrug resistance of compounds which accumulate in mitochondria, found in this study, is the result of similarity in drug transport mechanisms.

If energy-dependent mechanisms operate to allow concentration of QBQ and other drugs in mitochondria, it may be possible to design drugs to inhibit their selective concentration in mitochondria in the same way that verapamil and other compounds inhibit multidrug-resistance. Such inhibitors could be useful in several contexts, for instance by reducing mitochondria-mediated toxic side effects of the phthalanilides [4] as well as clinical agents such as doxorubicin [33]. We are currently investigating the possibility that mitochondrial accumulation of aromatic cationic drugs can be inhibited by non-toxic compounds such as verapamil in mammalian cells.

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