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CYTOTOXIC EFFECTS OF AMIODARONE AND DESETHYLAMIODARONE ON HUMAN THYROCYTES

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Abstract—Since recent *in vivo* evidence suggests that the benzofuran antiarrhythmic drug amiodarone has a direct toxic effect on the human thyroid gland, we have investigated the effects of both amiodarone and its metabolite desethylamiodarone on a novel immortalized functional human thyrocyte line (SGHTL-34 cells). Desethylamiodarone markedly reduced cell number as assessed from both DNA and protein content. Few cells were left after 24 hr exposure to 12.5 μg/ml; the concentration producing death of 50% of cells (EC_{50}) was 6.8 ± 1.1 μg/ml (mean ± SE, N = 15). Amiodarone was much less potent, producing a maximum decrease in cell number of approximately 25% at concentrations up to 50 μg/ml. The effect of desethylamiodarone was seen within 24 hr of culture. T_3 in concentrations up to 0.75 μg/ml had no effect on the action of amiodarone or desethylamiodarone on SGHTL-34 cells. Light microscopy demonstrated vacuolation of SGHTL-34 cells after 4-day culture with either the drug or its metabolite. Studies using primary cultures of human retroorbital fibroblasts demonstrated that the greater cytotoxicity of desethylamiodarone was not confined to thyrocytes. When SGHTL-34 cells were incubated with 2.5 μg/ml desethylamiodarone for 4 days, 71.7 ± 0.9% was taken up by the cells; there was no detectable conversion to amiodarone. Incubation of thyrocytes with 50 μg/ml amiodarone for 4 days resulted in the uptake of 80.1 ± 2.1% by the cells. In addition, 5.0 ± 0.1% of the amiodarone was converted to material with the same retention time as desethylamiodarone standard; of this material, 72.9 ± 2.8% was taken up by the cells. We conclude that desethylamiodarone, at concentrations near those found in the plasma of patients on long-term amiodarone therapy, exerts a direct cytotoxic effect on human thyroid cells in short-term culture. This effect may play a role in the aetiology of clinical thyroid disease during amiodarone therapy. We suggest that, since the effect is not restricted to thyrocytes, desethylamiodarone may play a role in the aetiology of amiodarone toxicity which occurs clinically in many tissues.

Amiodarone is a widely used drug effective in the management of a variety of arrhythmias including those, both ventricular and supraventricular, resistant to other drugs [1]. However, it has frequent side-effects involving a large number of tissues and these may limit therapy in many patients [1]. The effect of the drug on thyroid physiology is complex. Abnormalities of thyroid function tests are common in patients receiving amiodarone [2]; less frequently its use is associated with clinical hypothyroidism or hyperthyroidism which may occur by a variety of possible mechanisms [3]. Recent work has demonstrated direct effects of amiodarone on the human thyroid *in vivo*. Follicular destruction and fibrosis were noted in the thyroids of patients undergoing partial thyroidectomy for thyrotoxicosis presenting during amiodarone therapy [4]. These toxic changes were considered to be due to direct action by the drug or its metabolite, desethylamiodarone, both of which are concentrated in the thyroid gland [4–6], but there are no *in vitro* data available to substantiate this. In the present study, we have investigated directly the effects of both amiodarone and desethylamiodarone by examining their actions on a novel immortalized functional human thyrocyte line [7].

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MATERIALS AND METHODS

Materials. Except where indicated, amiodarone and desethylamiodarone (supplied by Sanofi) were dissolved in water at a concentration of 1 mg/ml by mixing and gently heating for several hours and stored in aliquots at −20°. Immediately before use the stock solutions were diluted into culture medium. The concentration in the stock and culture medium was checked periodically using high pressure liquid chromatography (HPLC, see below). Bovine thyrotrophin was purchased from Armour, gentamicin from Roussel, Ham's F12 and F10 from Flow Laboratories (Irvine, U.K.), *t*-butyl methyl ether (HPLC grade) from Aldrich Chemical Co. (Gillingham, U.K.); fenethazine was a gift from Rhone-Poulenc (Dagenham, U.K.) and $Na_2[^{51}Cr]O_4$ was purchased from Amersham (Amersham, U.K.). All other chemicals were from Sigma Chemical Co. (Poole, U.K.).

Thyrocytes. SGHTL-34 cells were cultured in 24-well plates (Falcon; initial plating density 10^4–10^5

cells/ml, 1 ml per well) in Ham's F12 medium with supplements [5% newborn calf serum (NCS), 16 μg/ml gentamicin, 2 mmol/l glutamine, 10 μg/ml insulin, 10 ng/ml somatostatin, 10 ng/ml glycine-histidine-lysine acetate, 3.6 ng/ml hydrocortisone, 5 μg/ml transferrin, and 40 μU/ml bovine thyrotrophin]. The plates were incubated in a humidified atmosphere containing 95% air:5% CO_2 at 37° for 2 days. The medium was replaced with 500 μl of the same medium containing amiodarone or desethylamiodarone at various concentrations in triplicate and the incubations continued for up to 4 days. For each time point the cells were washed once with 0.5 ml of 0.9% saline and stored at −20° until their content of protein and DNA was determined. In some experiments T_3, in concentrations up to 0.75 μg/ml, was incubated with thyrocytes in the presence or absence of amiodarone (25 μg/ml) or desethylamiodarone (2.5 or 7.5 μg/ml) for 4 days.

Fibroblasts. Human retroorbital fibroblasts were isolated by enzymatic digestion [8] of tissue obtained at strabismus operation. They were cultured in 24-well plates; initial plating density 10^3–10^4 cells per well in Ham's F10 with supplements (5% (v/v) neonatal calf serum, 16 μg/ml gentamicin, 0.12% (w/v) bicarbonate, and 2 mol/l *l*-glutamine]. They were incubated with amiodarone or desethylamiodarone in various concentrations for 4 days using the same protocol as for the SGHTL-34 cells.

Assay for cell growth. Protein was estimated using the method of Lowry *et al.* [9] using bovine serum albumin as standard. Estimates of total DNA content were determined using the fluorimetric method of Switzer and Summer [10] using calf thymus DNA as standard.

Assay for release of ^{51}Cr. Fibroblasts in 24-well plates were incubated with $Na_2[^{51}Cr]O_4$ in culture medium (20 μCi in 200 μl/well) for 4 hr, washed 4 times with culture medium and then incubated with various concentrations of amiodarone or desethylamiodarone in culture medium (1 ml/well) for 18 hr [11]. Cell-free medium (0.5 ml) was removed and counted (A); 100 μl of 10% Triton X-100 was added to the cells and the solubilized cells and remaining medium removed and counted (B). The percentage release for each well ($\%R_{exp}$) was calculated as 2A/(A + B). These data were then adjusted [11] to take account of background release by control cells ($\%R_{con}$) incubated without any drug (range 25 to 35%)

$$\text{cytotoxic index} = (\%R_{ex} - \%R_{con})/(\%R_{tot} - \%R_{con})$$

where $\%R_{tot}$ is the total releasable radioactivity from cells incubated with 2% Triton X-100 for 18 hr.

Assay for amiodarone and metabolite. Measurements of amiodarone and desethylamiodarone were made using the HPLC method of Flanagan *et al.* [12]. To estimate the uptake of the drug and metabolite, SGHTL-34 cells were incubated with known concentrations of each for 4 days. The medium and cells were separated; attached cells were washed and then treated with 300 μl of 1 M sodium hydroxide at 37° for 1 hr. In studies using desethylamiodarone, dead cells and debris were harvested from the medium by centrifugation at 12,000 *g* for 5 min, treated with 100 μl 1 M sodium hydroxide at 37° for 1 hr and assayed separately from the cells remaining in monolayer culture. Aliquots of both cells and medium were extracted in 200 μl *t*-butyl methyl ether before HPLC. Recovery was checked by parallel extraction of standards or the use of fenethazine as internal standard.

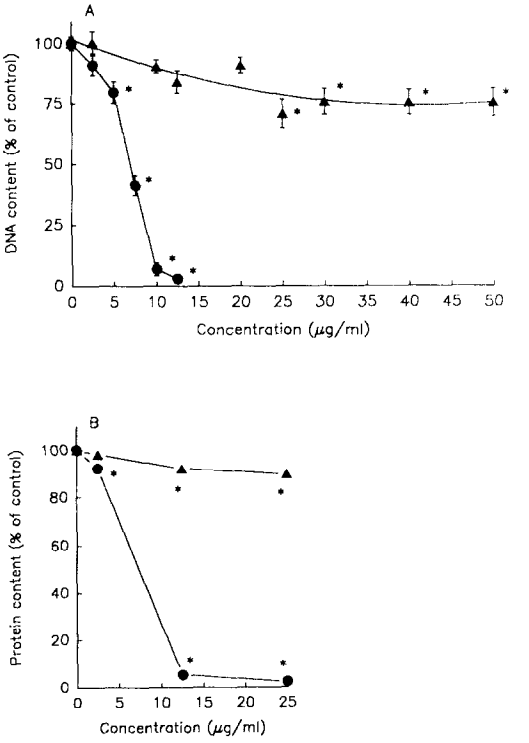

Fig. 1. (A) SGTHL-34 cells were incubated for four days in the absence of any drugs or with amiodarone (▲) or desethylamiodarone (●) in the concentrations shown. Results are expressed as a percentage of control DNA content (mean ± SE of four experiments performed in triplicate). (B) SGTHL-34 cells were incubated for 4 days in the absence of any drugs or with amiodarone (▲) or desethylamiodarone (●) in the concentrations shown. Results are expressed as a percentage of control protein content (mean ± SE of triplicate estimations from a typical experiment. Where SE bars are not shown they are encompassed by the symbol. *$P < 0.05$ compared with control (ANOVA and Tukey's HSD test).

Microscopy. SGHTL-34 cells were grown on coverslips and fixed in formol saline before staining with haematoxylin and eosin.

Statistics. Data were analysed using analysis of variance (ANOVA) and Tukey's honestly Significant Difference (HSD) test. Results are expressed as mean ± standard error of the mean (SE).

RESULTS

Amiodarone and desethylamiodarone clearly differed in their effects on the growth of SGHTL-34

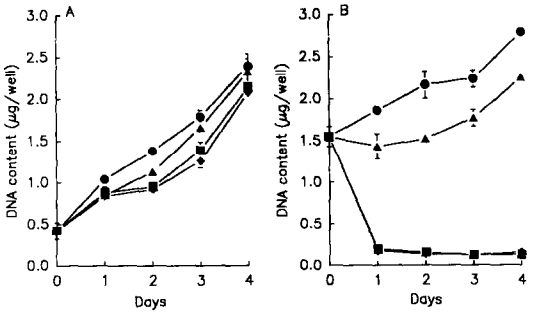

Fig. 2. SGHTL-34 cells were incubated for up to 4 days in the absence of any drug (●) or with amiodarone (A) or desethylamiodarone (B) in the concentrations (▲) 2.5 μg/ml (■) 12.5 μg/ml; (◆) 25 μg/ml. Results are expressed as mean DNA content per well (of triplicate observations from a typical experiment). Where SE bars are not shown they lie within the symbol.

cells (Figs 1 and 2). Desethylamiodarone markedly reduced cell number as assessed from both DNA and protein content. Few cells were left after 24 hr exposure to 12.5 μg/ml; the EC_{50} was 6.8 μg/ml. Recovery studies established that the effects were due to cell destruction and not decreased cell adhesion (data not shown). Amiodarone also reduced cell number with a maximum decrease of 30% of control values at concentrations up to 50 μg/ml (Figs 1 and 2). The effect of desethylamiodarone was seen within 24 hr of culture (Fig. 2).

Studies using primary human retroorbital fibroblasts demonstrated that the greater cytotoxicity of desethylamiodarone is not confined to thyrocytes (Fig. 3a). Using release of ^{51}Cr as a measure of cytotoxicity, desethylamiodarone was again shown to be considerably more active than amiodarone; however, unlike the protein and DNA assays, this technique apparently did not show considerable toxicity on the part of amiodarone (Fig. 3b). Comparison of the effects of amiodarone from stock solutions in either benzyl alcohol and Tween 80 (50 mg/ml, commercial supply for clinical use) or dissolved initially in a small quantity of 5% ethanol with data obtained using amiodarone made up in water alone demonstrated that the solvent had no effect on amiodarone induced ^{51}Cr release (not shown).

Neither the amiodarone nor the desethylamiodarone supplied contained significant cross-contamination as judged by HPLC (not shown). When SGHTL-34 cells were incubated with 2.5 μg/ml desethylamiodarone for 4 days, 71.7 ± 0.9% (mean ± SE of two experiments in duplicate) was taken up by the cells; no conversion to amiodarone was detected. Incubation of thyrocytes with 50 μg/ml amiodarone for 4 days resulted in the uptake of 80.1 ± 2.1% (N = 2) by the cells. In addition, 5.0 ± 0.1% of the amiodraone was converted to material with the same retention time as desethylamiodarone standard; of this material, 72.9 ± 2.8% was taken up by the cells.

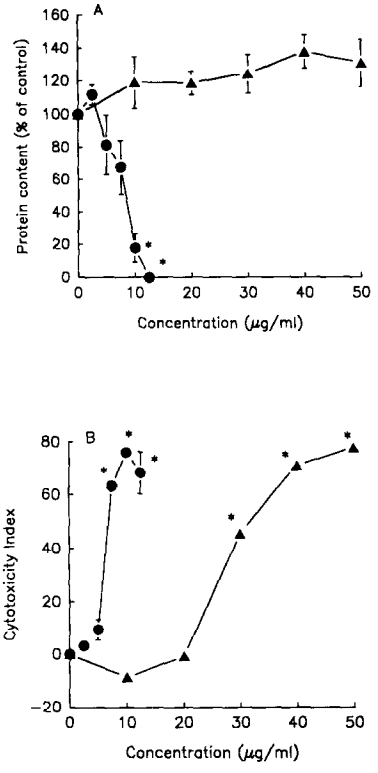

Fig. 3 (A) Human retroorbital fibroblasts were incubated for 4 days in the absence of any drugs or with amiodarone (▲) or desethylamiodarone (●) in the concentrations shown. Results are expressed as a percentage of control protein content (mean ± SE of a triplicate observations from three experiments). (B) Human retroorbital fibroblasts were preincubated with $Na_2[^{51}Cr]O_4$ and then incubated for 18 hr with amiodarone (▲) or desethylamiodarone (●) in the concentrations shown. Cytotoxicity index was calculated as described in the Materials and Methods section. Results are expressed as mean ± SE of duplicate estimations from two experiments. Where SE bars are not shown they are encompassed by the symbol. *$P < 0.05$ compared with control (ANOVA and Tukey's HSD test).

T_3 in concentrations up to 0.75 μg/ml did not modify the effect of amiodarone (at 25 μg/ml) or desethylamiodarone (at 2.5 and 7.5 μg/ml) on the DNA and protein contents of SGHTL-34 cells over 4 day incubations (not shown).

Light microscopy demonstrated that exposure of SGHTL-34 cells to the drug (25 μg/ml) or metabolite (7.5 μg/ml) resulted in vacuole formation (Fig. 4).

DISCUSSION

Amiodarone is a potent, clinically useful antiarrhythmic agent; both it and its main metabolite in man, desethylamiodarone, are amphiphilic and are characterized by accumulation and persistence within many tissues [5, 6]. It is associated with

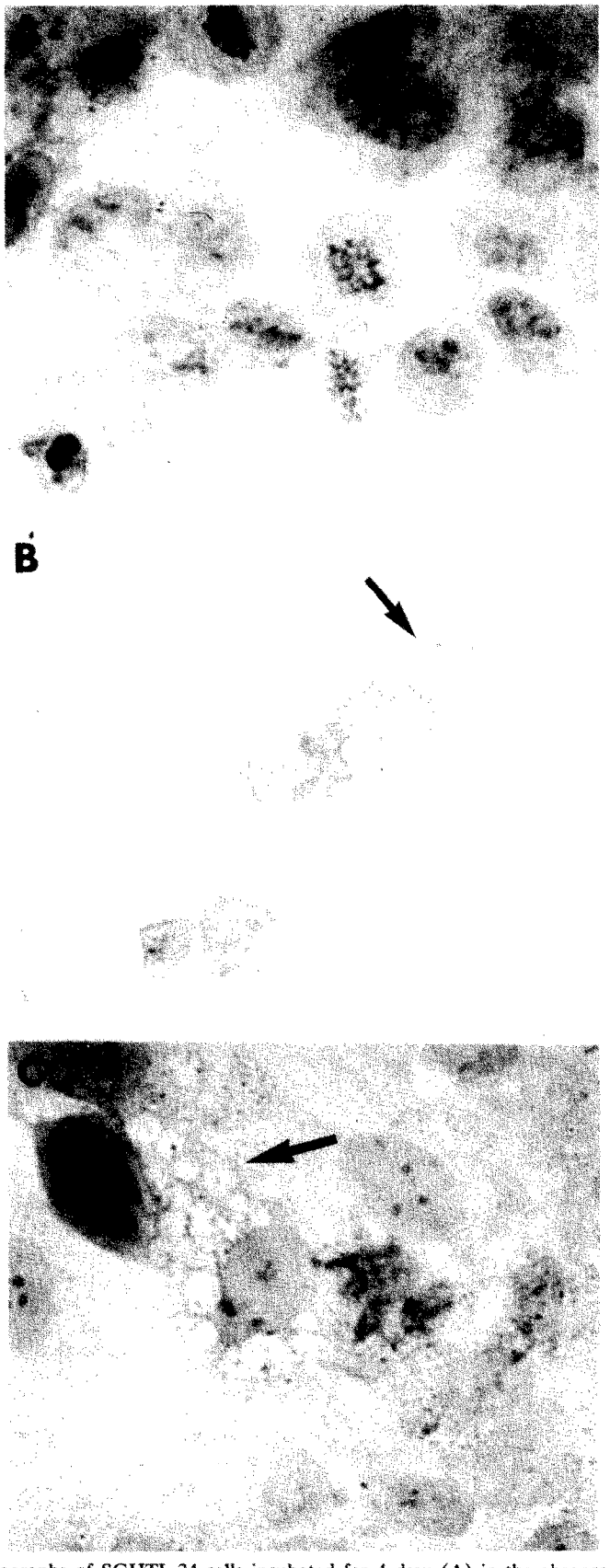

Fig. 4. Photomicrographs of SGHTL-34 cells incubated for 4 days (A) in the absence of any drug, (B) with amiodarone (25 μg/ml) or (C) with desethylamiodarone (7.5 μg/ml). Cells were grown on glass coverslips, fixed with formol-saline and stained with haematoxylin and Eosin.

frequent, troublesome side effects which limit its use. These occur in a large number of tissues and have similar histological features [13, 14] suggesting a common aetiology though both the agent and mechanism are unknown [1].

Amiodarone therapy exerts a number of effects on the pituitary–thyroid axis [15–19]; recent work [4] provides evidence that it produces thyroid damage which may exacerbate thyrotoxicosis. It is possible that the same process may play a role in the aetiology of hypothyroidism. In this regard it is interesting that, in the absence of underlying thyroid disease, amiodarone-induced hypothyroidism is reversible on cessation of therapy [20]. During our time course studies amiodarone did not decrease thyrocyte numbers below the values noted at Day 0 and, even at high concentrations of amiodarone, cell number was only reduced to approximately 75% of control values after 4 days. In contrast, desethylamiodarone exposure rapidly led to a dose-dependent reduction in cell number. This effect was demonstrable at concentrations approximating those in the plasma of patients on maintenance amiodarone therapy (say, 1–4 μg/ml [1]. However, two considerations prevent direct comparison of clinical data with our *in vitro* results. First, amiodarone is highly protein-bound in plasma and free drug concentrations may be lower. Second, thyrocytes in monolayer culture may be more sensitive to toxic effects than throcytes retained within the thyroid gland.

After incubation with thyrocytes for 4 days, approximately 5% of amiodarone was converted to material with the same retention time as desethylamiodarone on HPLC, suggesting that metabolism of amiodarone may occur within the human thyroid gland. Considering the much greater toxicity of desethylamiodarone, some of the effects of amiodarone on SGHTL-34 cells may result from this conversion.

Results using human retroorbital fibroblasts suggest that the effect of desethylamiodarone is not specific to thyrocytes. This is in keeping with the clinical observation of the multiplicity of amiodarone associated side effects and we interpret these data as demonstrating a direct cytotoxic effect of desethylamiodarone. The failure of amiodarone to reduce thyrocyte and fibroblast numbers conflicts with some other reports. For example, studies on the GH_3 cell line [21] showed that amiodarone decreased triiodothyronine stimulated [^{3}H]thymidine incorporation into these cells at concentrations as low as 3.4 μg/ml. Martin and Howard [11] examined the effect of amiodarone on bovine pulmonary artery endothelial cells *in vitro*. They showed that amiodarone increased ^{51}Cr release from these cells and used this as an index of cytotoxicity. We compared their ^{51}Cr technique with our DNA and protein analyses using, in some experiments, the same cells and drug solutions on the same day and found considerable discrepancies (contrast Fig. 3a with 3b). In agreement with Martin and Howard, amiodarone at 20–50 μg/ml caused the release of substantial amounts of ^{51}Cr after only 18 hr; however, there was virtually no effect on cell number 3 days later. Hence, ^{51}Cr release is not a reliable measure of cytoxocity in this case but is probably a reflection of the detergent-like property of the drug (Nussey and Johnstone, unpublished).

The mechanism of these cytotoxic effects on thyroid cells remains speculative. Our microscopical observations suggest that phospholipidosis [6, 13, 22] plays no role since cellular vacuolation was seen with both the drug and metabolite. Similarly our data on T_3 supplementation suggest that the toxic actions are independent of an inhibitory effect on the T_3 receptor which has been proposed to account for some of the effects of amiodarone in several tissues including the heart [16, 23]. We consider that the effect of desethylamiodarone is likely to be mediated at the plasma membrane. Both the drug and metabolite are amphiphilic; amiodarone is concentrated in membranes [24] and has direct effects on the fluidity of both experimental [25] and natural [26] membranes which is related to ionization [27]. Clearly, interactions with cardiac plasma membranes are involved in the electrophysiological actions on calcium [28] and sodium [29, 30] channels and β-adrenergic receptor [31, 32]. The only other study which, to our knowledge, has examined the effect of amiodarone on human thyrocytes in culture demonstrated [33] an exhibition of thyrotrophin stimulated cyclic AMP production at concentrations above 6 μmol/l (4.1 μg/ml). Since desethylamiodarone has similar effects on the heart [34, 35] it is likely that it too interacts in a similar way with the membrane though, to our knowledge, there are no experimental data for the metabolite. Certainly, the idea that desethylamiodarone toxicity is mediated by an effect on the plasma membrane is supported by the finding that it is more potent than the parent drug in increasing osmotic fragility and photosensitivity of human erythrocytes [36].

Why does removal of a single ethyl group from amiodarone cause such an increase in toxicity? We have found identical effects of amiodarone and desethylamiodarone (up to 100 μg/ml) on the surface tension of both water and culture medium (unpublished) suggesting that desethylation does not affect micelle formation over this concentration range. In addition, our data indicate that roughly similar amounts of the drug and its metabolite become cell-associated over the experimental period indicating that desethylation does not affect drug availability, for example by increasing binding to albumin. It is possible that removal of the ethyl group alters the charge on the amine, thus, affecting the interaction with membrane components.

In summary, we have demonstrated a direct cytotoxic effect of desethylamiodarone on a human thyroid cell line in short-term culture. This effect may play a role in the aetiology of clinical thyroid disease during amiodarone therapy. Since amiodarone toxicity occurs clinically in many tissues, the effect we have demonstrated in two cell types may be due to desethylamiodarone interaction with fundamental elements in cell membranes. Extrapolation of our results would suggest that modification of the amiodarone molecule to prevent formation of the metabolite may reduce the fre-

quency of side effects. Investigations into possible mechanism(s) of cell damage are currently underway.

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