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POTENTIATION OF CB 1954 CYTOTOXICITY BY REDUCED PYRIDINE NUCLEOTIDES IN HUMAN TUMOUR CELLS BY STIMULATION OF DT DIAPHORASE ACTIVITY

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Abstract—The toxicity of CB 1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] towards human cells was greatly enhanced by NADH (when foetal calf serum was present in the culture medium) and by nicotinamide riboside (reduced) (NRH), but not by nicotinate riboside (reduced). Co-treatment of human cells with CB 1954 and NADH resulted in the formation of crosslinks in their DNA. The toxicity produced by other DNA crosslinking agents was unaffected by reduced nicotinamide compounds. When caffeine was included in the medium, a reduction in the cytotoxicity of CB 1954 occurred. The toxicity experienced by human cell lines after exposure to CB 1954 and NADH was proportional to their levels of the enzyme DT diaphorase NAD(P)H dehydrogenase (quinone), EC 1.6.99.2. It is concluded that NRH, which we have shown to be a co-factor for rat DT diaphorase (Friedlos *et al.*, *Biochem Pharmacol* **44**: 25–31, 1992), is generated from NADH by enzymes in foetal calf serum, and stimulates the activity of human DT diaphorase towards CB 1954.

CB 1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide†] is an exceptionally potent anti-tumour agent capable of curing the rat Walker 256 carcinoma [1]. Its effectiveness against this tumour system was far superior to that of cisplatin, melphalan or chlorambucil, agents in routine clinical use. CB 1954, a monofunctional alkylating agent becomes both more reactive towards DNA [2] and difunctional in cells of the Walker tumour, where it induces DNA interstrand crosslinks [3]. The bioactivation of CB 1954 in Walker cells involves the reduction of its 4-nitro group to a 4-hydroxylamino group [4] by the enzyme DT diaphorase [NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2)] [5]. The dose of CB 1954 required for the same degree of kill is about 10,000 times less in cells able to perform this conversion than in cells that cannot [3]. DT diaphorase is thus an exploitable enzyme for inducing selective toxicity.

However, the human form of DT diaphorase metabolizes CB 1954 about seven times slower than does rat DT diaphorase [6]. Thus even those human cells that are high in DT diaphorase are insensitive to CB 1954. This explains why CB 1954 (although successful in the treatment of the rat tumour) was unsuccessful in the treatment of human tumours (Dr E. Wiltshawe, unpublished clinical trial). In view of the proven success of CB 1954 in the rat system, it would be highly desirable to be able to enhance the activity of human DT diaphorase towards CB 1954. This might re-create its remarkable anti-tumour activity in man.

We have previously observed that when NADH (itself completely non-toxic) was added to a non-toxic dose of CB 1954, a 60% kill was seen in V79 cells [4]. This cell line is now known to be very low in DT diaphorase [2]. We wished therefore, because of the potential value of CB 1954, to establish by what mechanism this effect occurred. We also wished to discover whether this potentiation of CB 1954 toxicity would be more dramatic in cells with higher levels of DT diaphorase and whether it also occurred in human cells.

MATERIALS AND METHODS

Chemicals. Chemicals were supplied by Sigma unless otherwise indicated. Nicotinate and nicotinamide ribosides were produced enzymically from their commercially available mononucleotides as described previously [2]. Nicotinate riboside was chemically reduced as described previously [7]. [^{3}H]-CB 1954 was supplied by Amersham International. CB 1954 was supplied by Prof. M. Jarman (ICR).

Cells and growth conditions. MRC-5 (an SV40-transformed normal human fibroblast) [8], MAWI (a signet-ring colon carcinoma) [9] and MCF-7 (a breast carcinoma) [10], all grew in Dulbecco's modified Eagle's medium (DMEM)-supplemented with 10% foetal calf serum (FCS). HeLa cells were grown in monolayer minimal essential medium (MMEM) with 10% FCS. HFL/M (a normal non-transformed human foetal lung cell) [11] was grown in Ham's F12 with 15% FCS. RT112 (a transitional

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† Abbreviations: CB 1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; NRH (nicotinamide mononucleoside—reduced), dihydronicotinamide riboside; PBS, phosphate-buffered saline; FCS, foetal calf serum; DMEM, Dulbecco's modified Eagle's medium; MMEM, monolayer minimal essential medium.

cell carcinoma of the bladder) [8] was grown in Ham's F12 with 10% FCS.

Cell survival assays. Volumes of recently harvested cells (1 mL; 2×10^5/mL) were treated with agents as indicated for 2 hr and survival determined by a colony-forming assay. Experiments were conducted both in serum-containing growth medium, and in serum-free medium. To add caffeine at <60 mM final operating concentration, the cells were pelleted, and then resuspended into medium containing the caffeine.

Determination of DNA interstrand crosslinking. [^{14}C]Thymidine-labelled MCF-7 cells were treated in fully complemented growth medium with CB 1954 (5 or 50 μM) plus NADH (500 μM) for 20 or 2 hr, respectively, plus a 3 hr post-treatment incubation period to maximize crosslinking [2, 12]. After harvest the cells were washed with phosphate-buffered saline (PBS), and mixed with ^{3}H-labelled control cells. Cell suspensions were gamma irradiated (5 Gy) and analysed by alkaline elution and by sedimentation in alkaline sucrose, as described previously [13].

Determination of intracellular NADH. Flasks (80 cm^2) of near-confluent MAWI cells (containing about 5×10^6 cells) were exposed to NADH (500 μM), CB 1954 (50 μM), or both NADH and CB 1954, for 2 hr. The cells were harvested by trypsinization, washed in PBS and resuspended in ice-cold PBS (10 ml), and the cell count determined. The cells were re-pelleted, and re-suspended in ice-cold PBS (1.8 ml) to which was added lysis solution (0.2 ml)(KOH 0.5M; 36% w/v CsCl; in 50% aqueous ethanol). The lysate was passed through a 0.2 μm filter and the concentration of NADH determined by HPLC as described previously [14].

CB 1954 uptake by cells. Replicate flasks (80 cm^2) of near-confluent MAWI cells (about 5×10^6 cells) were exposed to ^{3}H-labelled CB 1954 (50 μM; 20 Ci/mmol) in the presence or absence of NADH (500 μM). Cells from pairs of flasks were harvested, washed seven times in ice-cold PBS, and the cell number determined. The final cell pellets were dissolved in Soluene-350 tissue solublizer (Canberra-Packard) (1 mL), and the amount of radioactivity determined by liquid scintillation counting. The amount of CB 1954 (as indicated by the presence of tritium) in the washed cell pellets was expressed per cell.

DT diaphorase content vs survival studies. Cells from near confluent flasks (80 cm^2) were harvested, suspended in medium and counted. Sufficient cells (10^6) were withdrawn to assay the effects upon survival of CB 1954 (10 or 25 μM) plus NADH (50 μM) as above. The remaining cells were assayed for cytosolic DT diaphorase content as before, using menadione as substrate, cytochrome *c* as terminal electron acceptor and NADH as co-factor [6]. Results were expressed per mg of total cytosolic protein as determined using a commercial protein assay kit (Biorad). Units are nmoles cytochrome *c* reduced per minute.

RESULTS

Effect of NADH on the toxicity of CB 1954 to human cells

Evidence of the enhancement by NADH of CB 1954 toxicity in a human cell was obtained in the MAWI cell line. CB 1954 (50 μM) alone was non-toxic, but when NADH (50 μM) was also present, survival decreased to 0.2% (Fig. 1). Nicotinamide mononucleoside (reduced) (NRH) produced a similar effect. Nicotinate riboside (reduced) produced no enhancement of CB 1954 toxicity. Neither NADH, nicotinate riboside (reduced) nor NRH alone had any effect upon the survival of MAWI cells at these concentrations (data not shown). A fixed dose of NADH (50 μM) reduced the dose of CB 1954 required to reduce MAWI cell survival to 1.0% by 40-fold (Fig. 2). The toxicities of cisplatin, chlorambucil and melphalan towards MAWI cells were unaffected by co-treatment with 50 μM NADH

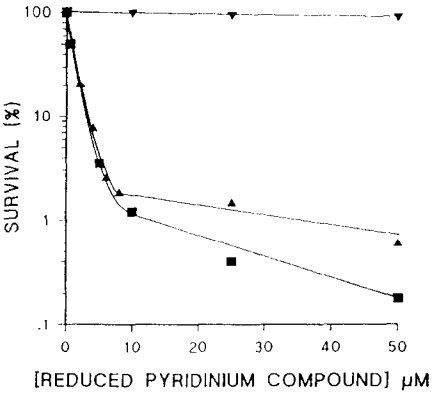

Fig. 1. The effect of NADH (■), NRH (▲), and nicotinate riboside (reduced) (▼) upon the survival of CB 1954-treated MAWI cells. The cells were harvested and exposed in suspension for 2 hr at 37° to 50 μM CB 1954 plus doses of reduced pyridinium compound as indicated, and then assayed for colony-forming ability.

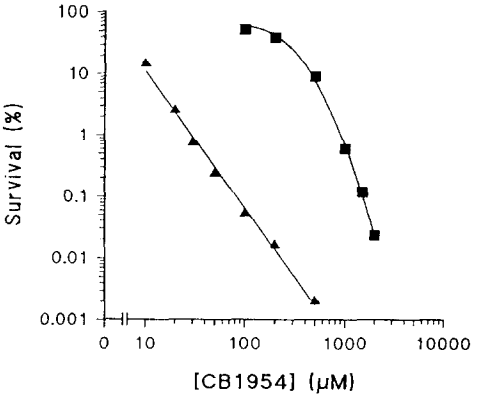

Fig. 2. The effect of CB 1954 upon the survival of MAWI cells in the presence (▲) or absence (■) of NADH. Cells were incubated in NADH (50 μM), or not, plus the doses of CB 1954 for 2 hr at 37° as indicated, and then assayed for colony forming ability. A logarithmic ordinate is used to accommodate the widely differing dose ranges of CB 1954 on one graph.

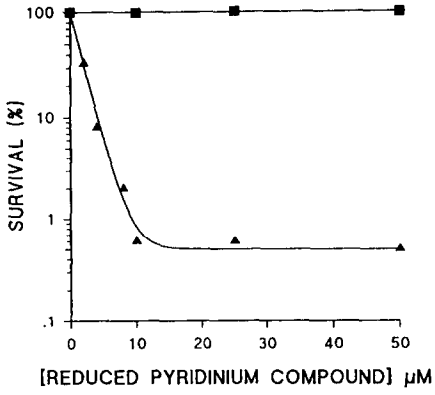

Fig. 3. The effect of NADH (■) or NRH (▲) on the survival of CB 1954-treated MAWI cells in the absence of FCS. The cells were harvested and exposed in suspension for 2 hr at 37° to various doses of NADH or NRH and 50 μM CB 1954 in DMEM without a FCS supplement.

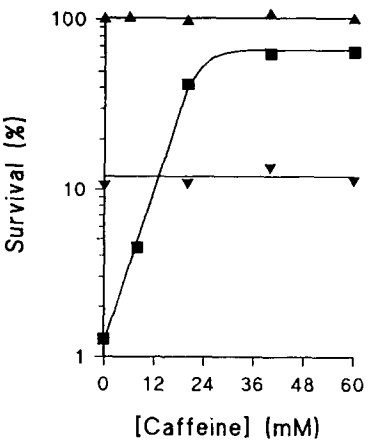

Fig. 5. The effect of caffeine upon the survival of CB 1954 plus NADH- or cisplatin-treated MAWI cells. The cells were harvested and exposed in suspension for 2 hr at 37° to either nothing (▲), 50 μM CB 1954 plus 50 μM NADH (■), or 50 μM cisplatin (▼), together with doses of caffeine as indicated, and assayed for colony forming ability.

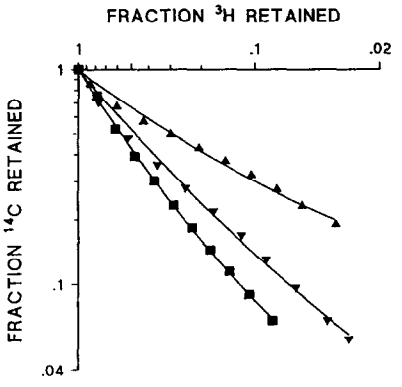

Fig. 4. The formation of crosslinks in the DNA of MCF-7 cells treated with CB 1954 plus NADH. [^{14}C]DNA-labelled cells were treated on monolayer with either nothing (■), or 5 μM CB 1954 plus 500 μM NADH for 20 hr (▲) or 50 μM CB 1954 plus 500 μM NADH for 2 hr followed by a 3-hr post-treatment incubation at 37° (▼). The cells were then harvested, mixed with ^{3}H-labelled untreated cells, irradiated (5 Gy) and analysed by alkaline elution.

(data not shown). When serum was excluded from the cell-treatment medium, no effect was seen of NADH on CB 1954 toxicity, whereas the effect of NRH was unaltered (Fig. 3).

Effect of CB 1954-plus-NADH on the elution of DNA from toxically affected cells

DNA crosslinking assays could not be conducted in the MAWI cells because these cells cannot incorporate radiolabelled thymidine into their DNA. The MCF-7 breast carcinoma cell that was also known to be high in cytosolic DT diaphorase [6] did incorporate labelled thymidine, and was thus suitable for crosslinking assays. The DNA from treated cell cultures eluted more slowly than did that from cells of the untreated culture, indicating the presence of crosslinks (Fig. 4). Crosslinking values of 0.44 and 1.58 crosslinks per 10^9 Da of DNA were seen for cells treated with 50 μM CB 1954 for 2 hr (followed by a 3 hr post-treatment incubation), and those treated with 5 μM CB 1954 for 20 hr, respectively. The unirradiated elution profiles (data not shown) provided no indication of DNA strand-breakage accompanying the crosslinking following these treatment protocols. Alkaline sedimentation analysis of the same DNAs produced similar results (data not shown).

Effect of an inhibitor of DT diaphorase on NADH potentiation of CB 1954 toxicity

Caffeine reduced the toxicity of CB 1954 (50 μM) plus NADH (50 μM) to MAWI cells. Survival rose from 1% in the absence of caffeine to about 70% when 40 mM caffeine was present during the 2 hr exposure period. There was little further reduction of toxicity with a dose of 60 mM caffeine (Fig. 5). The caffeine was present only for the 2 hr that the cells were exposed to drug, and had no effect upon the survival of MAWI cells (Fig. 5). Cisplatin (50 μM, 2 hr) reduced survival to 10%, but caffeine (60 mM) produced no modification of this toxicity (Fig. 5).

Effect of NADH upon intracellular levels of CB 1954 and of CB 1954 upon intracellular levels of NADH

MAWI cells were exposed to [^{3}H]CB 1954 in the presence or absence of NADH, and the amount of ^{3}H in the cells was monitored after 2 hr of continuous exposure. In the absence of NADH an accumulation of 18 pmol CB 1954 per cell was seen. In the presence of NADH, this value reached 26 pmol CB 1954 per cell.

Control MAWI cells were seen to contain 1.8 fmol of NADH per cell, while incubation for 2 hr in the presence of NADH (500 μM), CB 1954 (50 μM), or

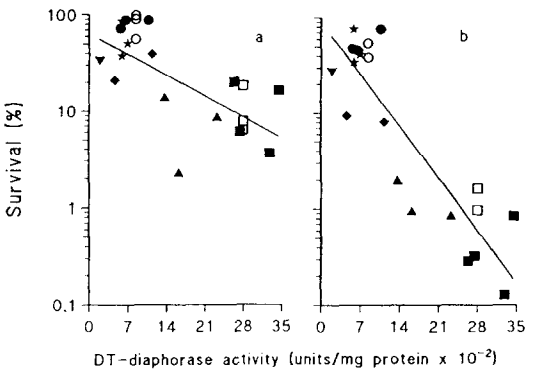

Fig. 6. The influence of levels of cytosolic DT diaphorase on the survival of six human cell lines following exposure to CB 1954 plus NADH (filled symbols) or NRH (open symbols). MAWI (■,□), MCF-7 (▲), MRC-5 (◆), HFL (▼), RT112 (★) and HeLa (●,○). Cells were harvested and assayed for cytosolic DT diaphorase, and exposed in suspension for 2 hr at 37° to 10 μM CB 1954 plus 50 μM NADH or NRH, (a) or 25 μM CB 1954 plus 50 μM NADH or NRH (b) and then assayed for colony-forming ability. The results are from independent experiments.

of both, yielded values of 1.2, 0.82, and 1.1 fmol per cell, respectively.

Effect of cellular DT diaphorase levels on NADH potentiation of CB 1954 toxicity

The survival of six human cell lines exposed to NADH (50 μM) and either 10 μM CB 1954 (Fig. 6a), or 25 μM CB 1954 (Fig. 6b) was inversely proportional to their DT diaphorase content. The toxicity was greater at the higher dose of CB 1954. Regression analysis indicated a minimal ($F_q(1,35) > 100$) chance that either line did not differ from zero, and a less than 2% chance that they did not differ from each other. Substitution of NRH for NADH produced similar results.

DISCUSSION

We previously observed that NADH slightly potentiated the toxicity of CB 1954 to Chinese hamster V79 cells [4], which have very low levels of cytosolic DT diaphorase [2]. We now show that NADH significantly potentiates the toxicity of CB 1954 to human tumour cells, which have much higher levels of DT diaphorase. There are several indications that DT diaphorase is mediating the toxicity of CB 1954-plus-NADH in human cells, as it does that of CB 1954 alone in rat cells.

First, crosslinking was observed in MCF-7 cells following exposure to CB 1954-plus-NADH. A dose of 5 μM CB 1954 for 20 hr produced greater crosslinking than a dose of 50 μM for 2 hr, consistent with a time-dependent activation occurring. This suggests that (as in Walker cells) the toxicity was due to the reduction of CB 1954 to its 4-hydroxylamino (crosslinking) species by DT diaphorase.

Second, there is no effect of NADH on the toxicities of the direct-acting drugs melphalan, chlorambucil and cisplatin. Thus, NADH does not produce a toxic synergy with all DNA crosslinking agents, regardless of their mechanism of crosslinking. This again suggests that NADH is affecting selectively the activation step of CB 1954.

Third, the possibility that NADH or CB 1954 was altering each other's intracellular concentration was investigated and disproved.

Fourth, an enhancement of the toxicity of CB 1954 (equal to that seen with NADH) is seen with NRH (see below), but not with the closely related chemical nicotinate riboside (reduced). This further implicates DT diaphorase, since NRH is a cofactor for the reduction of CB 1954 by DT diaphorase, whilst nicotinate riboside (reduced) is not [7].

Fifth, an inhibitor of DT diaphorase partially reversed the NADH-enhanced toxicity of CB 1954 towards human cells. We have demonstrated previously that caffeine is an inhibitor of DT diaphorase and is capable of alleviating the toxic effects of CB 1954 in Walker cells [12]. Ideally, as in our previous study, the DT diaphorase-specific inhibitor dicoumarol would have been used. However, as has been discussed previously [12], in cell culture dicoumarol is much less potent at protecting against the cytotoxic effects of CB 1954 than would be predicted by its inhibition constants with respect to pure DT diaphorase. Higher doses of inhibitor were required in the present experiments, corresponding to the higher concentrations of CB 1954 used, and under these conditions dicoumarol (at its maximum solubility) had little effect. This is probably a reflection of the competitive aspects of these inhibitors to the cofactor and substrate binding sites of DT diaphorase. To enable higher concentrations of the inhibitor to be attained, the more soluble caffeine, rather than the more specific dicoumarol had to be employed. A dose-responsive alleviation of the toxicity of CB 1954-plus-NADH occurred, again suggesting that the NADH is enhancing the ability of human DT diaphorase to reduce CB 1954. The possibility of a non-DT diaphorase-specific effect of caffeine is contra-indicated by the lack of an effect on the toxicity of cisplatin.

Sixth, the degree of toxicity experienced by human cell lines exposed to CB 1954-plus-NADH was proportional to their cytosolic DT diaphorase levels.

These six observations strongly argue that NADH is indeed enhancing the toxicity of CB 1954 by stimulating the extent of its reduction by human DT diaphorase. This phenomenon is dependent upon the presence of FCS in the medium, whereas the similar effect produced by NRH is not, and we have recently shown that enzymes in FCS hydrolyse NADH to yield NRH [14]. We have also shown that NRH is a co-factor for DT diaphorase, but not for another enzyme examined [7] and it is unlikely to be a co-factor for the majority of enzymes that are obligate for either NADH or NADPH. It would appear therefore that NADH (in the presence of serum) is enhancing the rate of reduction of CB 1954 by providing NRH, an additional DT diaphorase-specific co-factor.

The mechanism of this potentiation is not clear.

Perhaps the rate of reduction of CB 1954 in human cells by DT diaphorase is limited by availability of co-factor. NRH may be effective in countering this, because it is specific for DT diaphorase, and unavailable (and therefore unaffected) by the ubiquity of other oxidoreductases that are obligate for NADH or NADPH. It should be noted that even in the presence of NRH, the cytotoxicity of CB 1954 in human cells is still much less than in rat cells of equivalent DT diaphorase levels. This emphasizes the intrinsic differences in the abilities of the human and rat forms of DT diaphorase to reduce CB 1954.

The relationship between the toxicity experienced by a cell type and its level of DT diaphorase emphasizes that DT diaphorase is potentially a source of selectivity in cytotoxic chemotherapy. The experience of the Walker tumour would argue that this selectivity can be beneficial in the treatment of tumours. This may be because some tumour cells have elevated levels of DT diaphorase compared with their normal tissue of origin. This has been reported for human colon carcinoma cells [15], hepatoma cells [16], and breast, colon, liver and lung [17], although the reverse was true for human kidney and stomach [17], and for mouse lung [18]. It may also be because the normal tissue distribution of DT diaphorase directs CB 1954 toxicity away from tissues that are usually sensitive to conventional cytotoxic chemotherapy. Thus preliminary studies show that in the Walker tumour-bearing rat that bone marrow was the tissue with the lowest levels of DT diaphorase (Friedlos, Biggs and Jones, unpublished results). This would explain why the limiting toxicity of CB 1954 in rats was not haematological, as would usually be seen with alkylating agents.

In summary, the lack of success of CB 1954 as an anti-tumour agent in humans is because of the relative inactivity of human DT diaphorase towards CB 1954. We have shown that this inactivity can be surmounted in cell culture by addition of NADH (because of production of NRH), resulting in enhanced cytotoxicity of CB 1954. Future work will establish whether this can be translated into an anti-tumour effect in humans as useful as that originally seen in rats.

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