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THE PROPERTIES OF TOTAL ADDUCTS AND INTERSTRAND CROSSLINKS IN THE DNA OF CELLS TREATED WITH CB 1954

EXCEPTIONAL FREQUENCY AND STABILITY OF THE CROSSLINK

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Abstract—CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide†) becomes, upon bioactivation, a difunctional alkylating agent. It can be up to a 100,000-fold more cytotoxic in cells that are able to bioactivate it than in those that cannot. This increase in cytotoxicity is much greater than would be predicted from the conversion of a monofunctional alkylating agent to a difunctional one. We now show that the interstrand crosslink formed in the DNA of CB 1954-sensitive cells has some unusual properties. In Walker cells, which are able to activate CB 1954, the interstrand crosslink is the major adduct and can constitute up to 70% of the total adducts. These crosslinks are only poorly excised, as are those produced in V79 cells (which are themselves unable to activate CB 1954) by co-culturing them with Walker cells. Also, CB 1954 is approximately 10-fold more reactive toward the DNA of Walker cells than V79 cells. These observations may explain the extent of the increase in cytotoxicity accompanying the bioactivation of CB 1954.

CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide†) is a monofunctional alkylating agent. Following bioactivation, it becomes difunctional and capable of producing DNA interstrand crosslinks [1]. This bioactivation proceeds in two stages and has been elucidated in cells of the Walker 256 rat tumour.

The 4-nitro group of CB 1954 is first reduced to a 4-hydroxylamino group [2] by the enzyme NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2) [3] commonly known as DT diaphorase. The 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide undergoes a further (non-enzymic) reaction with *S*-acetylthioesters and appears to form 4-(*N*-acetoxy)-5-(aziridin-1-yl)-2-nitrobenzamide. This is the heterodifunctional molecule capable of reacting directly with DNA to form interstrand crosslinks [4].

Walker, and other rat cell lines with high levels of DT diaphorase are sensitive to CB 1954 [5]. Human cells that contain high levels of DT diaphorase are much less sensitive to CB 1954 [5]. This is because CB 1954 is a much poorer substrate for the human enzyme than for the rat form [5]. Treatment of cells which are unable to perform this bioreduction (and are therefore insensitive to CB 1954) with 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide resulted in crosslink formation and cytotoxicity similar to that experienced by Walker cells [5].

The increase in cytotoxicity observed in cells able to bioactivate CB 1954 corresponds to about 10,000–100,000-fold the dose (when compared with cells unable to perform this conversion) [1]. This is considerably greater than that previously associated with a mono- to di-functional transition. We have therefore investigated the changes in reactivity accompanying CB 1954 activation and the properties of the resultant crosslinks.

MATERIALS AND METHODS

Preparation of [^{3}H]*CB* 1954. [^{3}H]5-Chloro-2,4-dinitrobenzamide (Amersham International, Amersham, U.K.) was prepared by catalytic exchange with tritiated water and purified by silica gel chromatography. The tritiated intermediate was reacted with aziridine to give [^{3}H]CB 1954. This was purified by normal phase HPLC. The procedure yielded [^{3}H]CB 1954 of sp. act. 26.8 Ci/mmol. This was diluted with unlabelled CB 1954 to give the desired specific activities.

Cells and labelling conditions for crosslink analysis. Chinese hamster V79 cells were grown in minimum essential medium and 10% fetal calf serum. Walker 256 tumour cells were grown in Dulbecco's modified Eagle's medium 10% horse serum/10 mM glutamine. They were radiolabelled by overnight growth in the presence of either [^{14}C]- or [^{3}H]-thymidine (0.1 and 0.5 μCi/mL, respectively; 50 mCi/mmol; Amersham) followed by a 4-hr label-free period.

Binding of [^{3}H]*CB* 1954 *to cellular DNA.* Walker or V79 cells (500 mL; 5×10^5/mL) were exposed to

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† Abbreviations: CB 1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; PBS, phosphate-buffered saline; WS, wild-type Walker cell; WR, derived Walker cell substrain; cisplatin, *cis*-diamminedichloroplatinum(II); carboplatin, *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum (II).

[^{3}H]CB 1954 (1 μM; 0.23 Ci/mmol or 10 μM; 0.023 Ci/mmol) for 6 or 24 hr. For excision experiments, Walker cells were labelled with [^{14}C]-thymidine, exposed to [^{3}H]CB 1954 (10 μM; 0.023 Ci/mmol) for 2 hr and resuspended in fresh medium. Cells were harvested at 0, 24, 48 and 72 hr after treatment. The cells were washed three times with PBS (50 mL) and flash-frozen at −70°. The pellet was lysed in 15 mL *p*-aminosalicylate mixture and extracted with 15 mL phenol solution [6]. The DNA was precipitated from the aqueous phase with 2-ethoxyethanol and washed three times with 70% aqueous ethanol. The DNA was re-dissolved in 5 mL 1% sodium acetate and digested in turn with RNase (10 mg/mL; 10 min; 37°) and proteinase K (10 mg/mL; 1 hr; 37°). After re-extraction the DNA was dissolved in magnesium chloride solution (1 mL; 1 mM) plus DNase I (10 μg/mL). The digests were assayed for ^{3}H and ^{14}C activity by liquid scintillation counting and for DNA content by UV absorbance. Tritium content per unit weight of DNA evaluated binding of CB 1954. ^{14}C Content assessed extent of DNA synthesis during a time-course.

Determination of DNA interstrand crosslinking. [^{3}H]Thymidine-labelled Walker or V79 cells were treated with CB 1954. After various periods they were mixed with [^{14}C]thymidine-labelled untreated cells and analysed for DNA interstrand crosslinks. Both sedimentation in alkaline sucrose and alkaline filter elution were used, as described previously [7].

Conditions for co-culture experiments. Adherent V79 cells ([^{3}H]thymidine-labelled) were overlaid with Walker (WS) cells and treated with CB 1954 (10 or 20 μM) for 24 hr. The WS cells were rinsed off and the V79 cells harvested and re-seeded into replicate flasks. At intervals cells were collected and mixed with [^{14}C]thymidine-labelled, untreated cells. They were analysed for DNA interstrand crosslinks by alkaline filter elution.

Determination of crosslink stability in aqueous solution. [^{3}H]Thymidine-labelled Walker cells were exposed to CB 1954 (10 μM; 2 hr), harvested and their DNA extracted. The CB 1954-reacted DNA was dissolved in PBS containing [^{14}C]thymidine-labelled control DNA and incubated in the dark at 37°. At intervals aliquots were removed and the DNA analysed by sedimentation in alkaline sucrose.

Enzyme activity. DT diaphorase levels in WS, WR and V79 cells were assayed as described previously [3] employing menadione as substrate and cytochrome *c* as terminal electron acceptor.

RESULTS

Binding of [^{3}H]*CB* 1954 *to the DNA of* V79 *and Walker cells*

When Walker cells were exposed to [^{3}H]CB 1954, a time- and dose-dependent binding of ^{3}H to DNA was observed. Cells exposed to 1 μM CB 1954 displayed an approximately constant rate of binding of ^{3}H to DNA between 6 and 24 hr. A proportionate increase (at 24 hr) was seen when the dose was increased to 10 μM. V79 cells experienced about 10-fold less binding to DNA with all corresponding treatments (Table 1).

Table 1. Binding of CB 1954 to the DNA of Walker and V79 cells

	1 μM for 6 hr	1 μM for 24 hr	10 μM for 24 hr
V79	0.42	0.61	10.0
WS	3.51	9.53	94.4

Cultures of Walker and V79 cells were exposed to [^{3}H]-CB 1954 (1 μM for 6 and 24 hr, or 10 μM for 24 hr). The extent of binding of [^{3}H]CB 1954 to the DNA was assayed and expressed as nmol CB 1954 per g DNA.

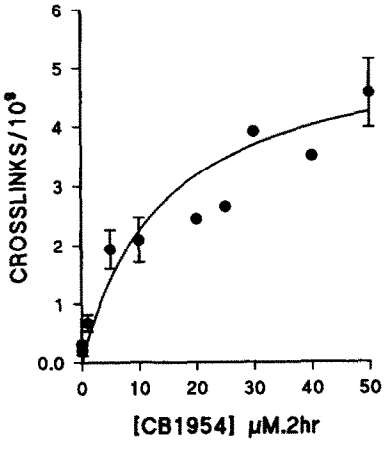

Fig. 1. Dose–response relationship between DNA interstrand crosslink formation and CB 1954 concentration in Walker cells. Cultures of [^{3}H]thymidine-labelled Walker cells were exposed to doses of CB 1954 for 2 hr before analysis of DNA interstrand crosslinking by alkaline filter elution. Allowance for the effects of drug-induced strand breaks was made. Error bars indicate standard error of the mean where three or more points were available as indicated. The line was obtained by a hyperbolic fit. Crosslinks were expressed per 10^9 Da of DNA.

Crosslinking of Walker cell DNA by CB 1954

A non-linear dose–response relationship exists between the concentration of CB 1954 and the extent of DNA crosslinking (Fig. 1). A hyperbolic fit to the data indicates a maximum degree of crosslinking of 5.4/10^9 Da of DNA and a dose of CB 1954 for half maximal crosslinking of 14.2 μM. When a dose of 1 μM is administered continuously, a linear increase in crosslinking with time is observed (Fig. 2). Also shown is the corresponding acquisition of overall adducts. The data indicate a crosslinking frequency of 71.5% at 6 hr and of 68.4% at 22 hr. Following a pulse dose of 10 μM, the crosslinking frequency immediately after the 2-hr pulse is only 4.8%, although this rises within 3 hr to 26%. This delay between maximum crosslink formation and time after removal of CB 1954 has been reported previously [8]. It is probably due to the fact that the initial reaction with DNA is monofunctional and is followed by a time-dependent reaction of the second

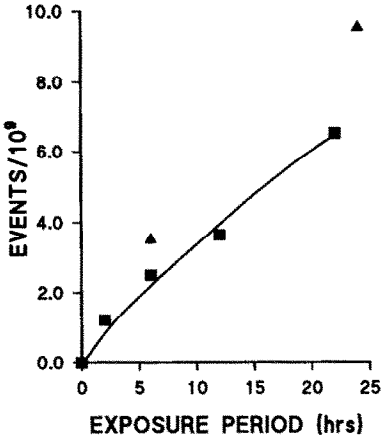

Fig. 2. The time-course of formation of DNA interstrand crosslinks and of total DNA adducts in Walker cells treated with CB 1954. Cultures of Walker (WS) cells were continuously exposed to either unlabelled CB 1954 or [^{3}H]CB 1954 (1 μM). Samples were removed at intervals and assayed for the presence of DNA interstrand crosslinks (■) by sedimentation in alkaline sucrose, and for total adducts (▲) by the presence of ^{3}H label. Both events were expressed per 10^9 Da of DNA.

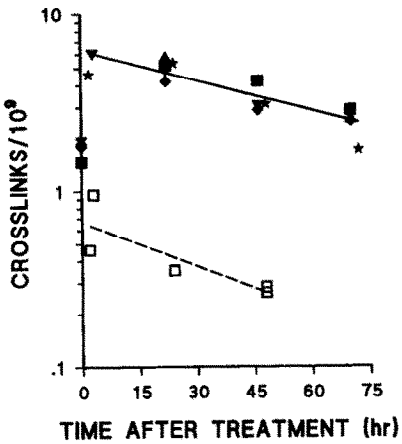

Fig. 3. Time-course of formation and loss of interstrand crosslinks in the DNA of Walker (WS and WR) and V79 cells treated with CB 1954. Cultures of WR or WS cells were exposed to CB 1954, 10 μM or 20 μM for 2 hr. V79 cells were co-cultured with WS cells for 24 hr in the presence of 20 μM CB 1954. The cells were resuspended in fresh medium. Aliquots were harvested at times as indicated, and assayed for DNA interstrand crosslinks by sedimentation in alkaline sucrose or by alkaline filter elution. By sedimentation: WR 10 μM (■); WR 20 μM (▲); WS 10 μM (▼); WS 20 μM (◆). By elution: WS 20 μM (★); V79 (□). Lines are regressions through all the WR/S data (unbroken) and V79 data (dashed). Crosslinks were expressed per 10^9 Da of DNA.

arm to produce the crosslinks. This phenomenon is also observed with other crosslinking agents such as platinum compounds [9, 10], nitrogen mustards [11, 12] and nitrosoureas [13].

Rate of loss of CB 1954-induced crosslinks from the DNA of Walker and V79 cells

Walker cells were exposed to CB 1954 for 2 hr and the crosslinks in their DNA analysed by alkaline sucrose gradient sedimentation (Fig. 3). No dose–response relationship was seen between 10 and 20 μM CB 1954. This is broadly consistent with what was observed using alkaline filter elution (Fig. 1). No difference in the amount of crosslinking was observed between the wild-type crosslink-sensitive Walker cell (WS) and a derived substrain (WR) of normal crosslink sensitivity. When the cells were incubated in drug-free medium for a further 72 hr, an initial average crosslinking level of 1.79/10^9 Da was seen to increase during 3 hr to 6.11/10^9 Da, as has been reported previously [8]. During a further 72 hr, a decline in crosslinking was observed indicative of a half-life for the crosslink of approximately 55 hr. V79 cells exposed to CB 1954 whilst in co-culture with WS cells for 24 hr acquired about 10-fold fewer crosslinks than did the WS cells. This crosslinking declined over a subsequent 48 hrs with a half-life of approximately 40 hr (Fig. 3).

Chemical stability of CB 1954-induced crosslinks

We wished to establish whether this half-life for the crosslink reflected a slow rate of DNA repair or whether this was the normal aqueous half-life of the lesion. DNA that had been extracted from CB 1954-treated Walker cells was incubated in PBS at 37°. It was seen that the DNA acquired breaks slowly, the treated DNA becoming broken slightly faster than the control. The degree of crosslinking however declined with a half-life of about 900 hr (Fig. 4). Thus, the rate of loss of crosslinks in aqueous solution was much slower than that observed for the repair of these lesions in cells.

Loss of overall adducts from the DNA of CB 1954-treated Walker cells

A biphasic time-course of loss of ^{3}H on DNA was observed (Fig. 5) when Walker cells were exposed to 10 μM [^{3}H]CB 1954 and then allowed up to 72 hr to repair adducts on DNA. An initial half-life of 30 hr was seen, fairly typical of aromatic substituents [14]. This proceeds until about 45 hr, at which point, a reduction in this rate is seen. Presumably this is the point at which most of the non-crosslink lesions have been lost. Also illustrated is the rate of loss of the crosslink expressed as a fraction of its highest value, 3 hr after treatment. Because excision of the non-crosslink adducts proceeds faster than that of the crosslink, the frequency of the crosslink rises from 26% of total adducts at 3 hr to 39.6% at 72 hr.

Enzyme levels

The levels of DT diaphorase in Walker cells were 1.87×10^4 U (WS), 1.3×10^4 U (WR), and only 50.1 U in V79 cells. Units were measured as nmol cytochrome *c* reduced per min per mg total cell protein.

DISCUSSION

The bioactivation of CB 1954 results in a vast

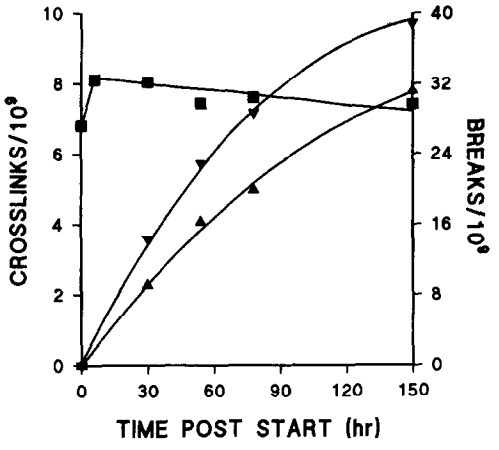

Fig. 4. Aqueous stability of CB 1954-induced crosslinks in DNA. [^{3}H]Thymidine-labelled Walker cells were exposed to CB 1954 (10 μM; 2 hr) and harvested, and their DNA extracted. The CB 1954-reacted DNA and [^{14}C]thymidine-labelled control DNA was co-dissolved in PBS, and incubated in the dark at 37°. At intervals aliquots were removed and the DNA analysed by sedimentation in alkaline sucrose. Changes in the molecular weight of control (▲) and treated (▼) DNA were expressed as accumulated breaks, and together with the extent of crosslinking of the treated DNA (■) were plotted against time. Crosslinks were expressed per 10^9 Da of DNA.

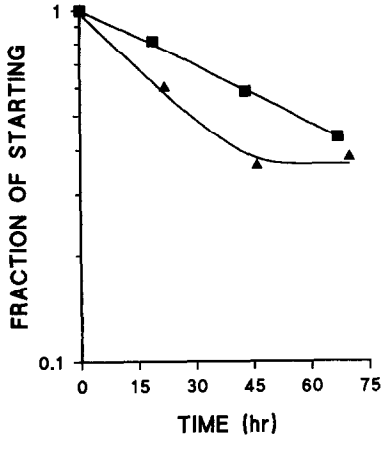

Fig. 5. Rates of loss of DNA interstrand crosslinks and of total DNA adducts from CB 1954-treated Walker (WS) cells. Cultures of [^{14}C]thymidine-labelled Walker (WS) cells were exposed to 10 μM [^{3}H]CB 1954 for 2 hr before resuspension in fresh medium. At intervals aliquots were harvested, the DNA extracted and assayed for specific activity of ^{3}H and ^{14}C. The ^{3}H was used to assess extent of binding of CB 1954 to DNA (▲), expressed as a fraction of the first measurement with the effect of DNA synthesis having been measured and compensated for by the change in ^{14}C specific activity. Also shown are data for the loss of crosslinks (■) derived from data as presented in Fig. 3 and expressed as a fraction of the highest value.

increase in its cytotoxicity. The resulting dose modification (as compared with V79 cells) is 10,000 (WR cells)- to 100,000 (WS cells)-fold [1, 15]. This is greater than would be predicted by a mono- to difunctional transition. Where monofunctional congeners of difunctional agents are available, as with half mustards and monofunctional platinum compounds, the dose modification for equitoxicity is seen to be around 50–200-fold [15, 16]. Our observations regarding DNA interstrand crosslink formation and their properties may explain why those cells able to bioactivate CB 1954 are so affected cytotoxically.

First, there is 10-fold more drug bound to the DNA of Walker cells than that of V79 cells. A commensurate increase in toxicity would be expected regardless of mono/difunctionality (although the increase in binding is probably a consequence of the bioreduction of the drug).

Second, the high frequency of the interstrand crosslink (up to 70%) could contribute to this extent of increase in toxicity. This frequency is much higher than that reported for other agents. For example, interstrand crosslinks represent 2% or less of the total DNA reactions of cisplatin [17, 18] and of carboplatin [10]. Co-treatment of both cisplatin and *trans*-diamminedichloroplatinum(II) treated cells by UV can double this frequency [19]. 8-Methoxy psoralen can produce crosslinking frequencies of up to 30% [20, 21] and novel tetrafunctional bis(platinum) complexes also appear to display high crosslink frequencies [22]. It has been argued that whilst the interstrand crosslink is of only minor importance to the overall cytotoxicity of (especially) cisplatin, it is in terms of molar efficacy a more intrinsically toxic lesion than are single-strand diadducts and monofunctional lesions [7]. An agent that produced a very high proportion of crosslinks would be expected to be more toxic than one that produced only a low frequency. The crosslink frequency varied with the treatment protocol used. This is probably due to saturation of the bioactivation stages at high doses of CB 1954. Previous work suggests that it is the second step that is rate limiting in those cells capable of generating the hydroxylamine [2].

Third, the low repairability of these crosslinks may be causing them to be even more intrinsically toxic than those induced by other difunctional agents. For instance, cisplatin-induced crosslinks are lost from the DNA of V79 and Walker (WS or WR) cells with half-lives of 13.3 [23] and 13.6 hr respectively [24], and from stationary human foetal lung cells with a half-life of 25 hr [25]. Shorter half-lives in the region of 4–12 hr are generally seen for crosslinks induced by organic agents such as nitrogen mustards [26–28] and nitrosoureas [29, 30]. The observed half-life of 55 hr of CB 1954-induced crosslinks in Walker cells may therefore be taken to be unusual. Wild-type (WS) Walker cells are inherently sensitive to crosslinks [15], however they are not defective in their excision [24]. Also, the non-crosslink-sensitive Walker cell (WR) [15] shows the same long half-life for the excision of CB 1954-induced crosslinks. These WR cells contain similar amounts of the activating enzyme DT diaphorase as

do WS cells and form the same numbers of crosslinks in their DNA on exposure to CB 1954. Furthermore, a similarly long crosslink half-life of 40 hr is seen in V79 cells. V79 cells are normally insensitive to CB 1954 (they have very low levels of DT diaphorase) but the co-culture technique allowed for the generation of CB 1954-induced crosslinks in the DNA of these cells (where they do not usually occur). Thus the long half-life of the CB 1954-induced crosslink may be taken to be a property of the lesion and not merely of the phenotype of the Walker cell.

The unusual properties of the CB 1954-induced interstrand crosslink suggests that this lesion is unlike those formed by other agents. The identification of the major interstrand crosslink lesion induced by CB 1954 will be the subject of a further publication. In brief, the 4-hydroxylamine (after activation as detailed previously [5]) reacts predominantly with the C8 position of deoxyguanosine. In DNA this would leave the aziridine function poised to react on the opposing strand (Davies, Jarman, Poon and Knox, unpublished data). Molecular modelling studies indicate that this second arm reaction will preferentially be on the O6 position of a deoxyguanosine on the opposite strand of DNA (Jenkins and Knox, unpublished data). Such a C8–O6 DNA interstrand crosslink would be unique and is not produced by other types of alkylating or platinating agents.

In summary, the unique properties of the CB 1954-induced crosslink may account for the dramatically increased toxicity of this compound following upon its bioactivation. Coupled with the selectivity of the bioactivation step by DT diaphorase, this explains why CB 1954 was so exceptionally effective as an antitumour agent in the rat and capable of curing the Walker carcinoma [31]. Unfortunately, CB 1954 is not readily reduced by the human enzyme [4] which precludes its use in the treatment of human tumours. However, the provenance of CB 1954 as an effective anticancer drug encourages further study. Analogues of CB 1954 may yet be made which are amenable to reduction by the human enzyme and thus exploit the unique chemistry of CB 1954 against human tumours. Also, it has been suggested that CB 1954 would be an appropriate prodrug for the ADEPT system in which an antibody-localized enzyme activates a prodrug to an active drug at the site of a tumour [32]. In systems proposed to date, the activation step works by virtue of a difunctional prodrug becoming more reactive. In the case of CB 1954, we have an example of a monofunctional prodrug becoming not only more reactive but also difunctional, and an example of difunctionality that is more cytotoxic than that produced by other agents. Thus in these respects, CB 1954 would appear to be an attractive ADEPT prodrug candidate.

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