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THE EFFECT OF DIBROMO-DULCITOL, DIEPOXY-DULCITOL AND VARIOUS NEW CYTOSTATIC HEXITOL DERIVATIVES ON THE METABOLIC ACTIVITIES OF NUCLEIC ACIDS AND PROTEINS—II

E. J. HIDVÉGI, JUDITH SEBESTYÉN,* L. D. SZABÓ, G. J. KÖTELES and L. INSTITORIS*

"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, 1775 Budapest 22, Hungary

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Abstract—The effects of nine antiblastic, 1,6-disubstituted hexitol derivatives were compared on DNA, RNA and protein synthesis. Each compound reduced ^{14}C-thymidine incorporation into the DNA in surviving rabbit bone marrow cell culture, *in vitro*. The rate of inhibition increased as a function of incubation time with the compounds and of their concentration. Among the hexitol skeletons carrying identical substituents dulcitol (galactitol) proved to be the most effective, D-mannitol was less and D-sorbitol the least effective. Comparing the reactive functional groups carried by identical hexitol skeletons, the dianhydride (diepoxide) derivatives were the most effective, dibromo derivatives were less and dimethanesulphonyloxy (dimesyl) derivatives the least effective. The inhibition of DNA synthesis by diiodo-D-sorbitol was anomalously strong. At higher concentrations the hexitols reduced precursor incorporation into RNA and protein of human tonsillar cells, *in vitro*. At relatively low concentration and short incubation times, however, enhancement could be observed. A good correlation was established between the rate of inhibition of DNA synthesis and the cytotoxic effect. Since the dianhydride derivative of hexitol was always more effective than its corresponding dibromo- or dimethane-sulphonyloxy derivative, the anhydrides may have formed from the latter *in vivo* and these are probably responsible for a large portion of the cytotoxic effect. The various hexitols, especially, dibromo-dulcitol and dianhydro-dulcitol, used with success in clinical practice have different properties *in vivo* (transport, transformation, target). In addition to DNA, the nuclear proteins may also be an important target site.

Investigations of the first alkylating hexitol derivatives acting on neoplastic diseases showed that the various functional groups on the hexitol skeleton influenced the nature of the cytostatic action. The first compound, Degranol®† [1], exerted a lymphotropic action [2, 3], characteristic of nitrogen mustards, and proved to be suitable in particular for the treatment of chronic lymphoid leukemia [4]. The next hexitol derivative, DMM [5, 6], contained the active methanesulphonyloxy (mesyl) group of myleran (busulphan). This compound strongly inhibited myelopoiesis, and resembled myleran. It has been successfully used clinically in the treatment of chronic myeloid leukaemia. In addition, it has tumor growth inhibitory properties [4, 7]. Considering this and its haematological effects, DMM represented a transition between the myleran-like and nitrogen-mustard-like pattern.

Institoris and coworkers studied analogues with groups related to the methanesulphonyloxy group in which bromine or iodine were coupled to the hexitols. They expected that the lipophylic carbon–halogen moiety on the polyhydroxy skeleton might ensure favourable transport characteristics; DBM [8] has been used clinically in the treatment of granulocytotic leukaemia [9–11]. The diastereomeric DBD [12] also proved to be an excellent myelotropic cytostatic [13–16]. Accordingly, both DBM and DBD are myelotoxic and, as regards their effect, are related to DMM.

It was concluded that changes of the substituents on the sugar alcohol skeletons or substitution of mannitol by dulcitol resulted in an appreciable change in the biological action. The present paper compares the effects of some recently synthetized cytotoxic hexitol derivatives on nucleic acid and protein syntheses of treated cells.

A further intention of these investigations was to determine whether the dihalogen–hexitol itself exerts some effect in the cell or whether it must first be transformed into an active compound within the cell. Davis and Ross [17] have already proposed for

* Cancer Research Laboratory, Chinoin Pharmaceutical and Chemical Works Ltd., Budapest, Hungary.

† Degranol ® = BCM = 1,6-bis(β-chloroethylamino)-1,6-dideoxy-D-mannitol.

Abbreviations and chemical structures of the compounds used: 1,6-dibromo-1,6-dideoxy-dulcitol, (or galactitol), DBD, Mitolactol®; 1,6-dibromo-1,6-dideoxy-D-mannitol, DBM, Myelobromol®; 1,6-dibromo-1,6-dideoxy-D-sorbitol, DBS; 1,6-dimethanesulphonyloxy-1,6-dideoxy-dulcitol (or galactitol), dimesyl-dulcitol, DMD; 1,6-dimethanesulphonyloxy-1,6-dideoxy-D-mannitol, dimesylmannitol or mannitol-myleran, DMM (or MM), Mannogranol®; 1,6-diiodo-1,6-dideoxy-D-sorbitol, DIS; 1,2-5,6 dianhydro-dulcitol (or -galactitol), diepoxy-dulcitol, DAD; 1,2-5,6 dianhydro-D-mannitol, diepoxy-mannitol, DAM; 1,2-5,6 dianhydro-D-sorbitol, diepoxy-sorbitol, DAS. (see Fig. 1).

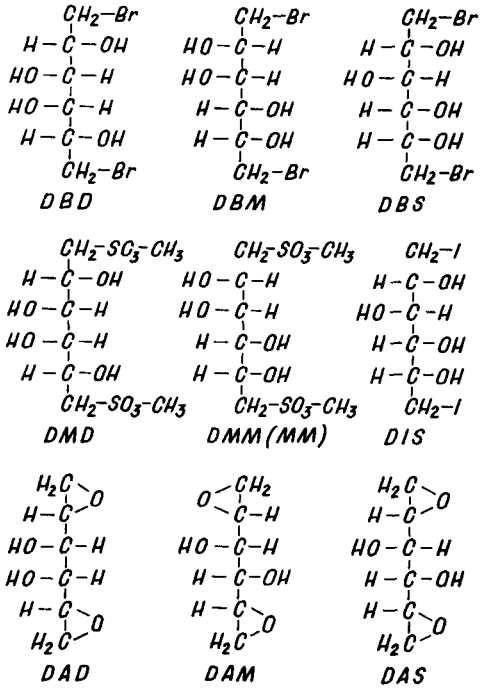

Fig. 1. Chemical structure of the 1,6-disubstituted hexitols used.

DMM that some reactive diepoxide (dianhydride) derivative is formed in the mildly alkaline physiological environment (pH 7.5). Since the formation of epoxide derivatives within the cell is theoretically possible both from DBM and DBD, this phenomenon was also studied. This type of investigation is important since experimental tumor research has suggested that as regards toxicity, tumor-inhibition and haematological effects, the dianhydro derivative of dulcitol, DAD, differs from DBD [18, 19] and also the clinical results obtained so far are promising [20].

Some new hexitol derivatives, DAS and DIS, not yet investigated biochemically were also studied. Each hexitol derivative studied, inhibited DNA synthesis in bone marrow cells in surviving cell cultures to different degrees depending on the concentration. In high concentrations they inhibited precursor incorporation into RNA and protein, while in low concentration they stimulated them.

MATERIALS AND METHODS

Preparation and incubation of bone marrow cell suspension. Bone marrow cell suspension was prepared according to Antoni *et al.* from the femora and tibiae of rabbits [21, 22]. Tyrode solution, pH 7.4, containing 0.1% glucose and 10% isologous serum was used for incubation. Ten ml of the suspension (10^7 cells/ml) was incubated in 100-ml Erlenmeyer flasks at 37°, without shaking, for 3 and 6 hr; 0.25 μCi ^{14}C-thymidine was added to each flask for the last 60 min. The non-treated control cells incorporated the ^{14}C-thymidine linearly during continuous labelling for at least 6 hr and therefore the comparative studies can be carried on for this long. At the end of the incubation the cells were quickly collected into cold saline, centrifuged and washed several times with cold saline.

Preparation and incubation of human tonsillar cell suspension. The cell suspension was prepared from human tonsillar cells according to the method of Piffkó *et al.* [23, 24]. Parker-199 medium, containing 20% heat-inactivated human serum of AB group, was used for incubation. Five ml of the suspension (10^7 cells/ml) was incubated in 100-ml Erlenmeyer flasks at 37° without shaking for various times up to 6 hr. To study RNA synthesis, 0.5 μCi of ^{14}C-uridine was added to each flask of tonsillar cell suspension. To label the proteins 1.0 μCi ^{14}C-glycine was added to each flask for the last 60 min of incubation. Incubation was terminated as described above for the bone marrow suspension.

Extraction of DNA, RNA, protein and determination of specific radioactivity. DNA and RNA were separated according to the modified Schmidt–Tannhauser method [25]. The DNA content was determined by Burton's reaction [26], the RNA content by orcinol reaction [22, 25]. The proteins were extracted according to the method of Siekevitz [27] and measured according to Lowry *et al.* [28].

Measurement of ^{14}C-radioactivity. Radioactivity was measured as infinitely thin layers in a Frieseke–Hoepfner methane-gas flow counter. Sp. act. values were expressed as cpm/μg DNA-P, cpm/μg RNA-P and cpm/mg protein.

Isotopes. Uniformly labelled ^{14}C-thymidine, sp. act. 20 mCi/mM, ^{14}C-uridine, sp. act. 500 mCi/mM and ^{14}C-glycine, sp. act. 109 mCi/mM were used. All the isotopes were obtained from The Radiochemical Centre (Amersham, England).

The hexitol derivatives. The hexitol derivatives were produced by Chinoin Pharmaceutical and Chemical Works Ltd., Budapest. Before use they were checked by melting point measurements. Since DAS could not be crystallized its epoxide content was checked according to Jarman and Ross [29]. The compounds were always freshly dissolved either in isotonic saline or Parker's Medium-199, at pH 7.4. The dianhydro compounds and DBS dissolved immediately at room temperature. The dissolution of DBM and DIS required 30 to 60 min at room temperature, while DBD and DMD could be dissolved only by stirring in a larger volume of medium for 1 hr at 37°. No more than 4–6% of each compound disintegrated under these conditions.

RESULTS

Incorporation of ^{14}C-thymidine into DNA of rabbit bone marrow cells. The inhibitory effect of the two new hexitol derivatives, DAD and DIS, was 2 to 3 times higher after incubation for 6 hr than after incubation for 3 hr, i.e. the inhibitory effect increased with prolongation of incubation time. The higher concentration of the compounds caused a higher degree of inhibition at the same time point.

Detailed comparative studies were performed after treatment for 6 hr with the various compounds. The effect of each compound was tested in a wide range of concentrations. Fig. 2A shows the effects of three dianhydro-hexitols (diepoxides) on ^{14}C-thymidine incorporation. All the dianhydro compounds strongly

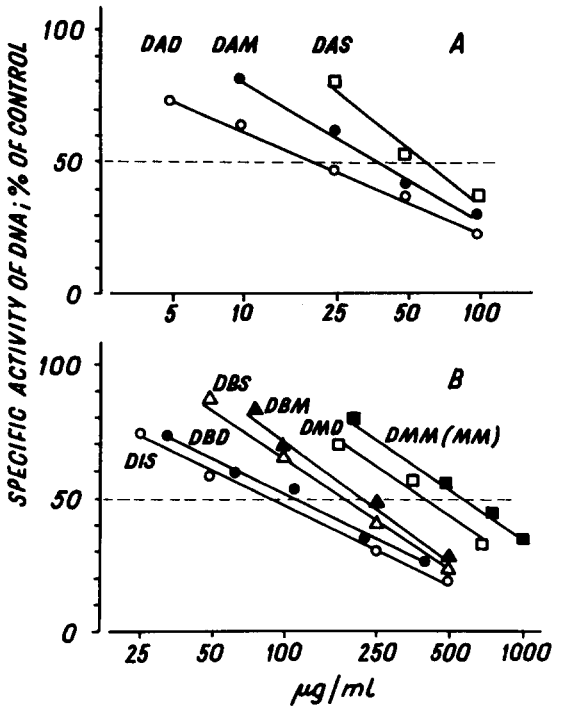

Fig. 2. Concentration dependent inhibitory effect of 1,6-disubstituted hexitol derivatives on ^{14}C-thymidine incorporation into DNA of rabbit bone marrow cells *in vitro*. Cells were incubated with the compounds for 6 hr from the beginning. Labelled thymidine was added to the cells 1 hr before stopping. (*A*) 1,6-dianhydro hexitols. (*B*) 1,6-dimethane sulphonyloxy hexitols.

inhibited the incorporation. The DAD was the most effective and DAS the least effective. Fig. 2B shows the effect of substituted hexitols. DIS was found to be the strongest inhibitor. Among the dibromo derivatives DBD was the most effective. Both DBS and DBM caused roughly the same degree of inhibition. Among the disubstituted hexitols the dimethanesulphonyloxy (dimesyl) derivatives were least effective.

The correlation between the effect on DNA synthesis and various reactive radicals of the molecule is most easily compared in the dulcitol series. As it is quite evident from Fig. 2, ^{14}C-thymidine incorporation was most strongly inhibited by the dianhydro derivatives DAD, the dibromo derivative DBD was less effective and the dimethanesulphonyl derivatives, DMD, the least. The ^{14}C-thymidine incorporation decreased linearly to the logarithm of the increasing concentration of each compound. It can be seen that if identical functional groups are located on various hexitols skeletons the curves will become convergent in the range of higher concentrations. However, if various functional groups are on an identical hexitol skeleton the curves run almost parallel; they are appreciably less convergent within the concentration range studied. As to the first type of curve, quite independently of its being plotted according to the logarithm of the concentration, e.g. in the range of 25% inhibition, the concentration of the less effective compound has to be increased 2.0 to 2.5-fold to achieve the same inhibition as the more effective compound. At about 75% inhibition this requirement involves an increase in concentration of only 1.3 to 1.5.

The degrees of efficiency of all the compounds can be compared on the basis of the inhibition of DNA synthesis in bone marrow cells, assessed by ^{14}C-thymidine incorporation. The concentration expressed in mM that reduced the rate of ^{14}C-thymidine incorporation by 50% was chosen as a basis for comparison. On comparing the stereoisomer hexitol skeletons, (Table 1) it was established that dulcitol is the most effective, D-mannitol and D-sorbitol the least effective. Dulcitol is 1.8 to 2.0 times more effective than D-mannitol, considering either the dibromo or the dianhydro derivative. Also DAM is 1.5 times more effective than DAS. If one takes the functional group the difference in inhibition is even more appreciable. The dianhydro derivatives are 2.7 to 2.9 times more effective than the dihalogens and the dihalogens 2.8 to 3.0 more effective than the dimethanesulphonyl derivatives. Among the sorbitol derivatives it is again the dianhydro derivative that is more effective than the dibromo derivative. On the other hand, the diiodo derivative behaves anomalously, since DIS is appreciably more active in this *in vitro* system than DAS. This anomaly will be discussed.

Incorporation of precursors into RNA and proteins of human tonsillar cells. Incorporation of ^{14}C-uridine into the RNA of tonsillar cells was stimulated by low concentrations of DAD, DBD and DBM, especially during short incubation times (Table 2). During the first 60 min of incubation this stimulation reached 30 to 50%. Stimulation ceased in proportion to the length of incubation time and inhibition developed

Table 1. Concentrations of hexitol derivatives inhibiting DNA synthesis in rabbit bone marrow by 50%

Compound	Chemical denomination	mM
DAD	1,2-5,6-dianhydro-dulcitol	0.136
DIS	1,6-diiodo-1,6-dideoxy-D-sorbitol	0.224
DAM	1,2-5,6-dianhydro-D-mannitol	0.274
DBD	1,6-dibromo-1,6-dideoxy-dulcitol	0.390
DAS	1,2-5,6-dianhydro-D-sorbitol	0.410
DBS	1,6-dibromo-1,6-dideoxy-D-sorbitol	0.650
DBM	1,6-dibromo-1,6-dideoxy-D-mannitol	0.730
DMD	1,6-dimethanesulphonyloxy-1,6-dideoxy-dulcitol	1.180
DMM (MM)	1,6-dimethanesulphonyloxy-1,6-dideoxy-D-mannitol	1.700

The mM values given in the Table are calculated from the data in Fig. 2.

Table 2. Effect of various hexitol derivatives on the incorporation of ^{14}C-uridine into RNA and of ^{14}C-glycine into proteins of human tonsillar cells *in vitro*

Compound	μg/ml	Specific activity (% of control) RNA				Protein			
		1 hr	1.5 hr	2 hr	3 hr	1 hr	1.5 hr	2 hr	3 hr
DAD	10	155	118	108	63	73	88	122	103
DBD	25	135	131	88	64	80	88	104	63
		3 hr		6 hr		3 hr		6 hr	
DAD	50	95		90		118		85	
DAD	100	109		45		118		60	
DAM	50	75		45		118		73	
DAM	100	70		45		103		63	
DAS	50	100		55		100		84	
DAS	100	100		65		92		75	
DBD	100	148		90		85		76	
DBD	150	122		76		80		68	
DBD	250	70		45		75		58	
DBM	250	122		80		104		90	
DBM	500	118		56		112		90	
DBS	250	60		65		105		85	
DBS	500	56		51		105		85	
DIS	250	58		67		89		80	
DIS	500	65		57		93		78	

gradually between 2 and 3 hr. Using relatively high concentrations of DAD, DAM and DAS the inhibitory effect was also time dependent. DBS and DIS reduced ^{14}C-uridine incorporation by about 40% during 3 hr and the prolongation of incubation for 6 hr did not increase this effect.

The incorporation of ^{14}C-amino acid into the proteins of tonsillar cells was initially slightly inhibited at low concentrations of DAD and DBD (Table 2). This inhibition was followed by slight stimulation at 2–3 hr with most of the hexitol derivatives. Stimulation was insignificant or even inhibition was observed with compounds containing sorbitol skeleton (DAS, DBS, DIS) or with DBD. After 6 hr and/or at higher concentrations of the compounds the amino acid incorporation regressed.

Under the *in vitro* conditions described the tonsillar cells did not show detectable DNA precursor incorporation for 6 hr [23, 24].

DISCUSSION

The cytotoxic hexitols exert the most prominent effect on DNA synthesis [30, 31]. Recently it has been established that ^{14}C-thymidine incorporation into satellite DNA is reduced more than into main band DNA after treatment of Yoshida ascites tumor cells with DBD and DAD *in vivo*[32]. Changes in ^{14}C-thymidine incorporation into DNA should be considered as changes in the rate of DNA synthesis and not as changes either in the nucleotide pool [21, 22] or of the inhibition of the polymerase enzyme and nucleotide triphosphate formation [33, 34]. As a result of DNA synthesis inhibition, 1 to 2 days after treatment with either DMM or DBM, the DNA content in the rabbit bone marrow drops to 30 to 50% [35, 47].

None of the hexitols appears to be particularly specific to any phase of the cell cycle. If administered in the sensitive phases of the cycle they cause premitotic block, mitotic delay and anomalies of mitosis [36], which is generally characteristic of the alkylating agents. DBD and especially DAD prevented ascites tumor cells from entering the S phase, and the latter was even more strongly inhibited [37, 38]. In synchronized cells, DBD acted on the S and G_1 phases [39, 40] while, as shown by age response tests for HeLa cells, the transition from S to G_2 and the M phase where most sensitive to DAD [41]. The results obtained by biochemical methods and by autoradiographic techniques are in good agreement with the studies with mustard gas [42].

These experiments did not determine whether the initial, increased incorporation of labelled uridine and amino acid into the RNA and protein, respectively, represented an enhanced synthesis of tonsillar cells. Part of this increase should be attributed to the enhancement of precursor transport through the cell membrane known for lymphocytes [43]. On the other hand the increased amino acid incorporation into protein is probably an indirect effect of the agents. It has been shown that DBM, DBD and DMM do not act directly on protein synthesis if measured in an *in vitro* cell-free ribosomal system [31].

Increase in RNA synthesis by mustard derivatives has been found previously [44, 46]. Treatment of Yoshida ascites tumor cells with DBD increased ^{14}C-orotic acid incorporation into the RNA *in vitro* [47], especially in the 20 S RNA fraction [48].

The question is whether the dihalogen and dimesyl hexitol derivatives themselves are acting in the cell or whether they are first transformed *in vivo*. The latter possibility was assumed first by Elson and Ross *et al.*, who postulated the formation of epoxide intermediates from disubstituted hexitols [2, 17, 29, 49, 50]. From the present experiments an essentially lower

concentration of dianhydride (diepoxide) hexitols inhibited DNA synthesis in bone marrow cells than of dihalogen or dimesyl derivatives. This supports the suggestion that under *in vivo* conditions, weak alkylating disubstituted hexitols gradually transform into strongly alkylating epoxide derivatives and the latter attack the target directly. This suggests that the dianhydro hexitols should be used. Under *in vivo* conditions, however, the situation is modified by at least two factors.

One of these factors is that the biotransformation of the disubstituted hexitols takes place through a number of intermediates. The biotransformation of DBM and DBD has been studied most intensively [51]. After hydrolysis, they might split so that they react directly with the target or, in slightly alkaline medium, form cyclic anhydrides. After DBD administration, besides the unchanged molecule, all the solvolytic intermediates were detected *in vivo*, among them the various bromo- and epoxide derivatives [52, 53]. It seems to be of interest that following the administration of DAD, unchanged drug could never be detected in tumor tissues, possibly because it reacted quickly with the nucleophilic sites of the tumor [54]. The various hexitols *per se* and their biotransformation products (epoxides) come into contact with many targets. Consequently, they may have multilateral effects. The early, epoxide-like cytotoxic effect of dibromo-hexites is due to drug that enters cells after transformation to the epoxide. The myleran-like effects are due to the dihalogen and dimesyl hexitols that have entered the cell intact [50]. Gradually, epoxides are also released from these and this might form an intracellular reserve for biological epoxide alkylations [55]. The earlier contradictions between the cytotoxic effect and alkylating ability of disubstituted hexitols appear to be reconciled by the fact that under *in vivo* conditions the compounds have a different transformation ability than *in vitro*, in aqueous medium.

The anomalously strong effect of DIS on DNA synthesis might be explained by its susceptibility for biotransformation. The sorbitol derivatives are cytotoxic also in cell cultures [56]. On transplanted tumours, however, they did not have a definitive inhibitory effect [56]. It may be assumed that the halogen derivatives of sorbitol (and xylitol) are more labile *in vivo* than the halogen derivatives of dulcitol or mannitol and are degraded by the detoxication mechanism of the whole organism.

The other factor that should be taken into account in the administration of hexitols is their different biotransports. The dibromohexitols that enter the organism are transported bound to albumin, while the epoxides formed from them are unbound [57]. The brain tissues absorb more DBD than DAD and the situation is quite the opposite for the gastrointestinal tract [58]. The accumulation and the elimination of active metabolite is different for the various derivatives [58]. The halogen and anhydride hexitols with different chemical structures have different transports, "fates", accumulation and elimination. Accordingly, the experimental antitumour and haematological effects are different [19, 20]. This widens and at the same time specializes their field of clinical application.

The mechanism of action of hexitols is based on alkylation since hexitol derivatives with alkylating ability below a certain degree are ineffective. DNA seems to be one of the most sensitive targets of the bifunctional hexitols in which they might form cross links between DNA strands. No cross links could be detected by DNA melting technique in the isolated DNA treated with hexitol derivatives *in vitro* [59]. However, because of possible methodological problems, the existence of the cross link should be examined by various methods. In fact, applying another method, a good correlation could be found between the formation of cross links and cytostatic effect of dihalogen hexitols [60].

Recently, instead of DNA, attention has been focused on the nuclear proteins as the potential point of attack of hexitols and in general, of alkylating agents. Pretreatment of the histones with hexitol derivatives prevented the formation of the corresponding nucleohistone with DNA [59]. More labelled DBD bound firmly to the histones and to the non-histone proteins and less to DNA [61]. The changed function of the altered nucleohistone is suggested by the increase of DNA-dependent RNA polymerase activity following *in vivo* DBD treatment [62]. These results suggest that the binding of alkylating antitumor compounds to nuclear proteins may bring more profound and permanent changes than the cross bond in DNA which may be eliminated by repair [42]. This binding to the nuclear proteins might form an essential component of the mechanism by which these agents inhibit the multiplication of the cell [63, 64]. The assumption of a single point of attack for the alkylating agents would be a simplification of the *in vivo* possibilities.

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