ANTICANCER RESEARCH *11:* 1077-1082 (1991)

Effect on Protein Synthesis and Cell Survival of the Benzaldehyde Derivatives Sodium Benzylidene Ascorbate (SBA) and the Deuterated Compound Zilascorb(^{2}H)

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Abstract. *Three different benzaldehyde derivatives (viz. ß-cyclodextrin benzaldehyde inclusion compound (CDBA), 4, 6-O-benzylidene-D-glucose (BG) and sodium benzylidene-ascorbate (SBA) have been shown to exert anticancer effects in patients without causing side effects. The anticancer effects are, however, variable and in many cases weak. In a previous study we showed that benzaldehyde with a deuterated formyl group gave rise to a greater protein synthesis inhibition than non-deuterated benzaldehyde. Based on this deuterated benzaldehyde we have synthesized an ascorbic acid acetal; 5,6-benzylidene-d_1-L-ascorbic acid (zilascorb(^{2}H). In the present paper we compare the effect of this drug with respect to cell inactivation and inhibition of protein synthesis in human cells cultured in vitro with that of benzaldehyde, BG and SBA. It is shown that zilascorb (^{2}H) is clearly the most effective of these drugs. The effect of zilascrob(^{2}H) is reversible in the sense that protein synthesis regains its normal level shortly (i.e. within 1h) after removal of the drug. Even after protracted treatment inducing a cell kill of more than 99%, the few survivors appear to be without damage after removal of the drug.*

Over the last decade derivatives of benzaldehyde have been tested as possible cancer chemotherapeutic drugs. The interest in these compounds originated from the work of Dr. Mutsuyuki Kochi and his coworkers, who showed a clinical effect in patients with solid tumours of mainly carcinoma type by two different benzaldehyde derivatives, CDBA (ß-cyclodextrin-benzaldehyde) (1) and BG (4, 6 benzylidene-D-glucose) (2). Although variable and in some cases small, the effect was found on many implanted tumours in mice (3-5). The positive clinical reports, together with our own finding of tumour necrosis after intra-arterial injection of BG in rats having chemically-induced hepatocellular carcinoma (6), stimulated further efforts to learn more about the benzaldehyde derivatives.

From experiments on human cells in culture we have shown that both benzaldehyde (7,8) and BG (9) induce a specific inhibition of protein synthesis resulting in reduced cell-cycle progression. It is not clear whether this effect is responsible for the antitumour effect. However, for our cells in culture, cell inactivation, as induced by BG, appears to be a secondary result of prolonged protein synthesis inhibition, since cell inactivation appears only after prolonged drug treatment and only when the drug dose is increased to a level where the rate of protein synthesis is little higher than that of protein degradation (9). A possible explanation is that cell deah follows from shortage of vital proteins.

More recent studies have indicated ways of improving the efficacy of the benzaldehyde derivatives. Firstly, clinical results published by Kochi have indicated that an acetal derivative of benzaldehyde and ascorbic acid, sodium benzylidene-ascorbate (SBA), is even more effective than BG (10, 11). Secondly, our own studies have indicated that the effectiveness of benzaldehyde itself as a protein synthesis inhibitor may be increased by exchanging the hydrogen atom in the formyl group with a deuterium atom (12, 13).

In the present study we have performed comparative studies between SBA and its deuterated analog zilascorb (^{2}H) sodium salt which has a deuterium atom at the acetal carbon (originating from benzaldehyde). We have also tested the deuterated compound with respect to its reversibility.

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Key Words: Zilascorb(^{2}H), deuterium, protein synthesis, cell culture.

Materials and Methods

Cells cultured in vitro. Cells of an established human line of cervicial carcinoma origin, denoted NHIK 3025, (14, 15) were cultivated in medium E2a (16 - 18) supplemented with 10% horse (Grand Island Biological Co., USA) and 20% human (prepared in the laboratory) serum. The cells are routinely grown as monlayers in plastic tissue culture flasks (Nunclon, Nunc, Denmark). The cells are kept in continuous exponential growth by frequent reculturing, *i.e.* every second and third day. Under growth conditions as described here, these cells have a median cell-cycle time of ≈ 18.5 h, with median G1, S and G2 durations of ≈ 7, ≈ 8 and 2.5 h, respectively. Mitosis lasts for about 50 min (17).

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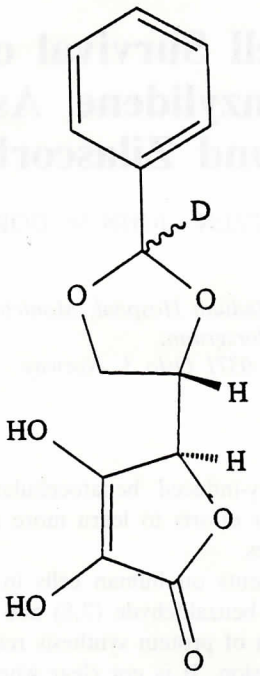

Figure 1. *Structure of 5, 6-Benzylidene-d_1-L-ascorbic acid indicating the position of the deuterium. The compound appears in the form of the two diastereoisomeres shown.*

Cell synchronization. Populations of synchronized cells were obtained from exponentially growing cells by collecting detached mitotic cells after a shaking procedure as previously described (17). Selections were performed repeatedly with 45 minutes intervals. Within 1 h the cells had divided and attached as doublets on the bottom of the flasks. Such synchronized cells have the same cell cycle and phase durations as exponentially growing cells (17). The entrance of the cells into mitosis as well as cell division was registered at various times after mitotic selection by observing approximately 100 cells within a delineated area in a tissue culture flask.

Cell survival. Before seeding, the cells were loosened from the tissue culture flasks by a mild trypsin treatment, then suspended as single cells. Appropriate numbers of cells were seeded in 25 cm^2 plastic flasks (Falcon 3002, Falcon, USA). After about 2 h in an incubator at 37° C (NAPCO, National, USA), the cells had attached to the bottom of the dishes. Drug treatment was then started by replacing the medium with medium having the appropriate drug concentration. After treatment the drug-containing medium was replaced with fresh, drug-free medium after a rinse. The flasks were incubated for 10 to 12 days before the cells were fixed in ethanol and stained with methylene blue. Cells giving rise to colonies containing more than 40 cells were scored as survivors (16).

Protein metabolism. The rates of protein synthesis and protein degradation were calculated as described previously (19). Briefly, cellular protein was labeled to saturation during a 2-day preincubation with [^{14}C]valine (Amersham; CFB 75, UK) of constant specific radioactivity (0.5 Ci/mol) prior to the experiment. This was achieved by using a high concentration of valine (1.0 mM) in the medium (20). At this concentration of valine, the dilution of [^{14}C]valine by intracellular valine and by proteolytically generated valine will be negligible (20), thus keeping the specific radioactivity at the constant level. The rate of protein degradation was calculated from the release of acid-soluble radioactivity to the medium during 2 h of incubation in medium containing 1.0 mM unlabled valine, thus prevening reincorporation of isotope (20). The rate of protein synthesis was

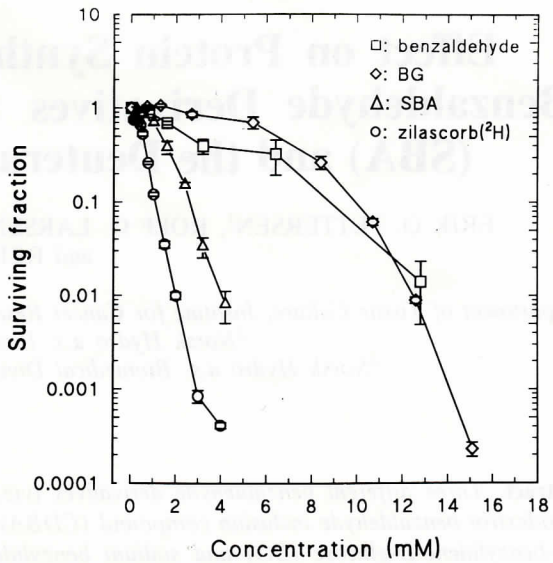

Figure 2. *Surviving fraction of NHIK 3025 cells after 24h treatment at 37° C with either zilascorb(^{2}H), SBA, BG or benzaldehyde. The latter two curves were redrawn from (9) and (7), respectively. The experimental points represent mean values of 5 replicate dishes with standard errors indicated as vertical bars.*

calculated from the incorporation of [^{3}H]valine (Amersham; TRK 533) of constant specific radioactivity (25 ci/mol, 1.0 mM). Both release and incorporation measurements were related to the total [^{14}C]radioactivity in protein at the beginning of the respective measurement periods and expressed as the percentage per h (19).

Chemical compounds. Zilascorb (^{2}H): This is the INN name (International Nonproprietary Name) for 5, 6-benzylidene-d_1-L-ascorbic acid (structural formula shown in Figure 1). Zilascorb (^{2}H) is prepared from deuterated benzaldehyde dimethylaceal and L-ascorbic acid as described earlier (12). The sodium salt of zilascorb (^{2}h) was used in all experiments.

SBA: 5, 6-O-benzylidene ascorbic acid sodium salt, (sodium benzylidene ascorbate).

BG: 4,6-O-benzylidene-D-glucose.

Benzaldehyde: This compound was purchased from Koch-Light Laboratories Ltd (UK), vacuum distilled in our laboratory, and stored under N_2.

Results

Exponentially growing cells were treated with various concentrations of either SBA or zilascorb (^{2}H) for 24h at 37. In Figure 2 the cell survival is shown as a function of drug dose. For comparison, cell survival as measured after similar treatment with benzaldehyde and BG is redrawn from previously published data (7). From these data, zilascorb (^{2}H) is clearly more effective in inactivating our cells than SBA. In fact, while the LD_{90} dose for zilascorb(^{2}H) is about 1 mM it is about 2.5 mM for SBA. In comparison, the LD_{90} dose for benzaldehyde and BG was shown to be around 9 mM for this treatment time, showing that both derivatives involving ascorbic acid are more efficient than benzaldehyde and BG in inducing cell inactivation.

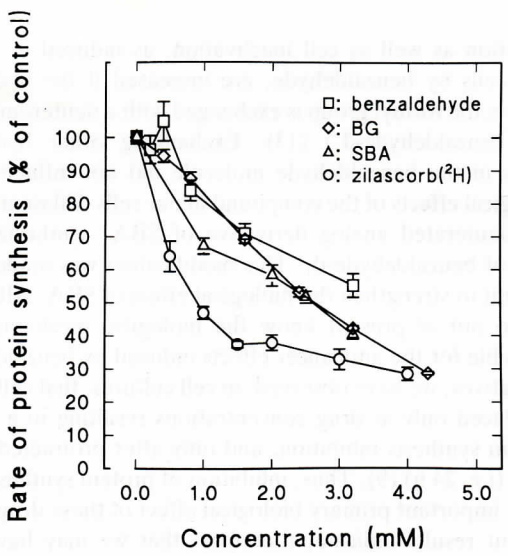

Figure 3. *The rate of protein synthesis relative to untreated controls of NHIK 3025 cells treated with either zilascorb(^{2}H), SBA, BG or benzaldehyde. The rate of protein synthesis was measured by the amount of [^{3}H]-valine incorporated during the first hour after start of drug treatment. Protein synthesis was meausred relative to the total amount of protein in the cells. The experimental points represent mean values of 3 to 5 replicate dishes with standard errors indicated as vertical bars.*

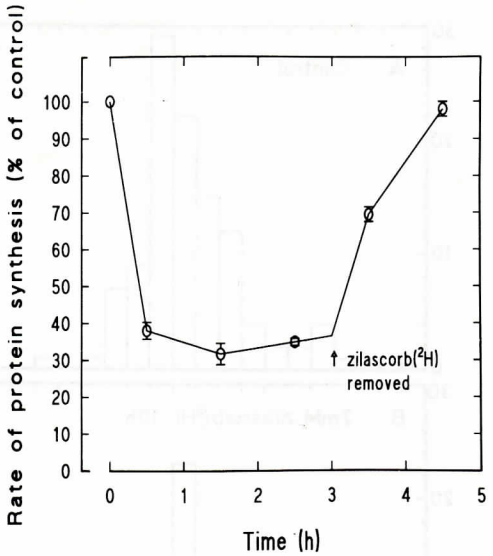

Figure 4. *The rate of protein synthesis relative to untreated controls of NHIK 3025 cells treated with 2 mM zilascorb(^{2}H) for 3h. Protein synthesis was measured during both treatment and the first 2h after zilascorb (^{2}H) was removed. Each measurement represent a 1h pulse with [^{3}H]-valine and the experimental points were plotted in the middle of this pulse on the time scale. The experimental points represent mean values of 3 replicate dishes with standard errors indicated as vertical bars.*

Table I. *The rate of protein synthesis and protein degradation in NHIK 3025 cells during treatment with zilascorb(^{2}H) and SBA.*

Treatment	Protein synthesis %/h	Protein degradation %/h
Control	5.71 ± 0.25	2.21 ± 0.08
1 mM zilascorb(^{2}H)	2.34 ± 0.15	2.16 ± 0.40
1 mM SBA	3.86 ± 0.03	1.81 ± 0.40

The effect of SBA and zilascorb(^{2}H) on the protein metabolism (*i.e.* protein synthesis and -degradation) of our cells was measured and compared with that induced by benzaldehyde and BG (Figure 3 and Table 1). As was earlier found with both benzaldehyde and BG (8, 9), both SBA and zilascorb(^{2}H) are effective inhibitors of protein synthesis with little or no effect on protein degradation. From Figure 3, however, it can be seen that the inhibition of protein synthesis is clearly greater with the deuterated compound zilascorb(^{2}H) than with the undeuterated compound SBA, and that both are more effective than benzaldehyde and BG.

One of the most striking features with the benzaldehyde derivatives so far tested by us is the reversible nature of their protein synthesis inhibiting effect. Since zilascorb (^{2}H) induced the strongest cellular effects of the drugs tested, it was of particular interest to test the reversibility of this drug. The data of one test is shown in Figure 4. In this experiment NHIK 3025 cells were treated with 2 mM of the drug for up to 3 h. The rate of protein synthesis was measured both during treatment and during the first two hours after treatment. Simultaneous measurement of cell survival showed no cell inactivation after this short treatment (data not shown). The results of Figure 4 show that the rate of protein synthesis regains its normal level shortly (*i.e.* in about 1h) after the drug was removed, although it was reduced to just above 30% of the normal value during treatment.

Other evidence of the reversible nature of zilascorb (^{2}H) is shown in Figure 5 representing colony-size analysis of NHIK 3025 cell colonies after treatment with zilascosb(^{2}H). The cells were treated for 10 h with either 2 mM or 10 mM zilascorb(^{2}H) and thereafter incubated for 12 days. Cell survival was found to be 23 and 0.7% respectively after treatment with the two different concentrations. Nevertheless, the colony-size analysis showed that mean colony diameter (analyzing randomly 110 colonies in each group) was 1.15 ± 0.05, 1.01 ± 0.05 and 0.86 ± 0.06 mm^2 for cells treated with no drug, 2 mM or 10 mM zilascorb(^{2}H) respectively. No morphologic difference could be seen between the cells of these different colonies. The cell density within the colonies was found to be about 1600 cells per mm^2 irrespective of treatment. Thus, the cell number per colony decreased from roughly 1800 in the control to 1400 in colonies formed by cells treatment with 10 mM zilascorb (^{2}H). This corresponds to a number of cell cycles of 10.9 in the control and 10.4 in the cells surviving 10 mM zilascorb (^{2}H). Thus, although no more than 0.7% of the cells treated with 10 mM for 10 h survive the treatment, these survivors have essential-

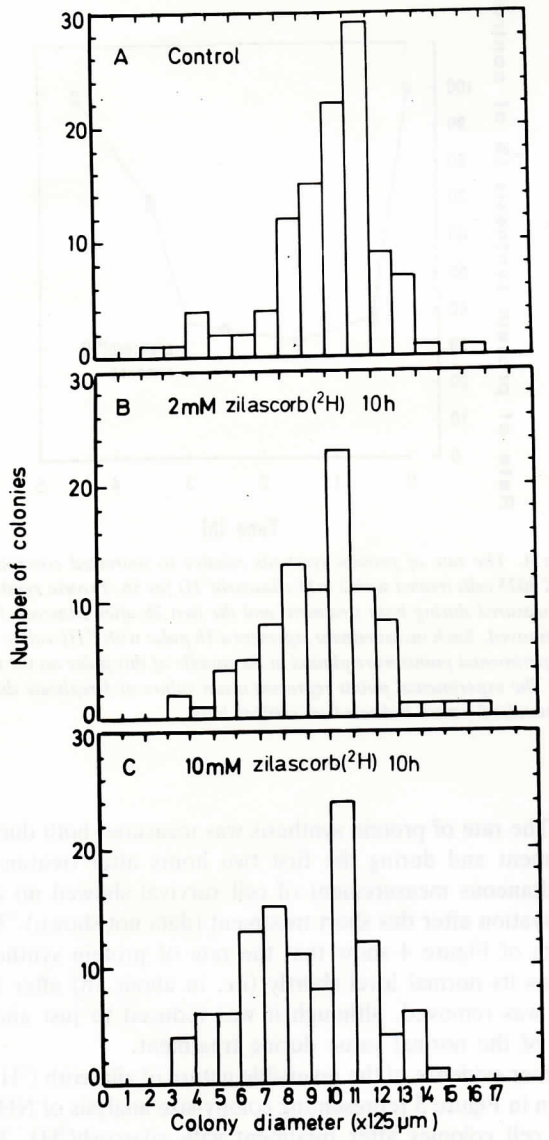

Figure 5. *Colony size histograms of colonies formed by single NHIK 3025 cells after 12 days of incubation. During the first 10h of incubation, the cells were either untreated (panel A) or treated with 2 or 10 mM of zilascorb-(^{2}H) (panel B and C respectively). Colony size was registered by inserting an occular having a grid system of squares in an inverted microscope. The side lengths of these squares equated 125 µm in the colonies. Colony diameters, therefore, were registered as multiples of 125 µm. The cell density per colony was also registered and found to be the same, ≈ 1600 cells per mm^2, in all groups.*

ly the same cell cycle time as the untreated cells after treatment. The small difference of 0.5 cell cycles is not more than the delay induced during the treatment itself when the control cells continues throughout the cell cycle while the treated cells are arrested.

Discussion

In a previous paper we have reported that protein synthesis inhibition as well as cell inactivation, as induced in NHIK 3025 cells by benzaldehyde, are increased if the hydrogen atom in the formyl group is exchanged with a deuterium atom (*i.e.* benzaldehyde-d_1) (13). Exchanging other hydrogen atoms in the benzaldehyde molecule did not influence the biological effects of the compound in our cells. Zilascorb (^{2}H) is a deuterated analog derivative of SBA, synthesized on basis of benzaldehyde-d_1. This modification was made in an attempt to strengthen the biological effect of SBA. Although we do not at present know the biological mechanism responsible for the anticancer effects induced by benzaldehyde derivatives, we have observed, in cell cultures, that cell death is induced only at drug concentrations resulting in a strong protein synthesis inhibition, and only after protracted treatment (*i.e.* 24 h) (9). Thus, inhibition of protein synthesis may be an important primary biological effect of these drugs. The present results indicate, therefore, that we may have succeeded in synthesizing a more efficient benzaldehyde derivative by deuteration of the molecule. Not only is the protein synthesis inhibition as induced by zilascorb(^{2}H) increased as compared with SBA (Figure 3), but this effect is also reversible (Figure 4) in the same manner as was the case with benzaldehyde (8) and BG (9). The reversibility of the drugs may be of importance in connection with the apparent lack of side effects seen following use of these drugs (1, 2, 21). It is, therefore, important to notice that the very small fraction of cells able to survive 10 mM zilascorb(^{2}H) for 20 h had a growth rate as well as a morphological appearance after treatment which was quite normal (Figure 4). This indicates that the surviving cells are left unharmed by the treatment, which is quite different from, for example, the situation following radiation treatment where even surviving cells usually have a reduced ability to proliferate.

The benzaldehyde derivative which has so far been most extensively investigated clinically is BG. This compound has been shown to induce anticancer effects in patients having different types of solid tumours with an overall response rate of more than 50% (1, 21). The effects are, however, in some cases weak or absent. In our hands BG was not effective in a phase II clinical trial including 14 patients with metastases from adenocarcinoma of colon and rectum (22). The patients were treated for 2 months, and it was concluded that 13 of the patients showed progressive disease during treatment, while 1 showed stable disease. However, in a study on rats with chemically-induced liver cancer we fond that over a period of just 10 days all tumours necrotized dramatically, one even completely, following daily intra-arterial injections of BG through a teflon canula inserted in the carotid artery (6). In a clinical study of SBA in patients with solid tumours of different origins Kochi reported a response rate comparable to that of BG (10).

Due to the variability in the clinical effect of the benzaldehyde derivatives, it is important to develop drugs that are more biologically potent but have the same type of effect as BG. The present results indicate that deuteration of the

acetal carbon of the ascorbate derivative of benzaldehyde has improved the biological efficacy of the compound.

Acknowledgements

The skillful technical assistance of Charlotte Borka, Ursula Prehn Hansen, Kari Skogen and Olav Tveitå is gratefully acknowledged. This study was supported by the Norwegian Cancer Society.

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Received January 28, 1991
Accepted March 1, 1991