Biochemical Pharmacology, Vol. 39, No. 2, pp. 309–318, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

REQUIREMENT OF A REACTIVE ALDEHYDE MOIETY FOR ALDEHYDE-MEDIATED PROTECTION AGAINST *CIS*-DICHLORODIAMMINEPLATINUM-INDUCED CELL INACTIVATION

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(*Received* 2 *March* 1989; *accepted* 16 *August* 1989)

Abstract—The effect of the aromatic aldehydes benzaldehyde and salicylaldehyde, the glucose-acetal derivative 4,6-benzylidene-D-glucose (BG) and the glucoside salicylaldehyde-β-D-glucoside (helicin) on cell inactivation induced by *cis*-dichlorodiammineplatinum (*cis*-DDP) was investigated using cultured human NHIK 3025 cells. Cell inactivation was measured as loss in the ability of single cells to give rise to macroscopic colonies following drug treatment. The fraction of cells surviving a 2 hr treatment with 10 μM *cis*-DDP increased from 0.012 ± 0.004 to 0.10 ± 0.03 when treatment was combined with at least 1 mM benzaldehyde or at least 0.2 mM salicylaldehyde. Of the two sugar-aldehyde derivatives only helicin protected cells from the inactivating effect of *cis*-DDP, although to a much lesser extent than either benzaldehyde or salicylaldehyde. While helicin retains the aldehyde moiety of salicylaldehyde, BG does not possess any free aldehyde group. Using synchronized cells we found these effects to appear in all phases of the cell cycle. Measurements of cell-associated platinum indicated that the degree of protection from the inactivating effects of *cis*-DDP by these aldehydes was related to the degree of reduced platinum accumulation. We conclude that this reduced accumulation may represent an inhibition of specific cell membrane uptake sites via Schiff based formation between membrane amino groups and aldehydes.

Benzaldehyde, at non toxic concentrations, reduces the cell inactivating effect of the chemotherapeutic drug *cis*-dichlorodiammineplatinum (*cis*-DDP†) when cultured cells are treated with both compounds in simultaneous combination [1]. Since the cell-associated platinum content is reduced after the combined treatment as compared to treatment with *cis*-DDP alone we have previously discussed the supposition that benzaldehyde appears to reduce cellular uptake or accumulation of *cis*-DDP by binding to cell membrane amino groups via a Schiff base reaction [2], thereby affecting a membrane-mediated uptake mechanism for *cis*-DDP. Benzaldehyde has been shown to bind to membrane protein of both *E. coli* [3] and SV 40-transformed cells [4], indicating that a reaction between benzaldehyde and cellular protein may be limited to exposed membrane proteins. Our supposition is supported by our findings that the structurally related aldehyde pyridoxal 5′-phosphate, also protects cells against *cis*-DDP-induced cell inactivation [5] although this compound cannot cross the cell membrane.

Although the above references represent an insight into the mechanism of action of benzaldehyde in general, a complete description of the protective effect is still lacking. Therefore, in the present report three related benzaldehyde derivatives (salicylaldehyde, helicin, and BG; Fig. 1), each with differing chemical structure and reactivity with cellular amino groups, are compared with benzaldehyde. The ability of these compounds to form Schiff bases may be directly related to their ability to modify *cis*-DDP cytotoxicity. The absence of an aldehyde moiety, as in BG, would produce a compound unable to form a Schiff base. Bulky substituents, such as the glucose moiety in helicin, may reduce the formation of a Schiff base due to steric hindrance. Finally, substitution at the 2-position of benzaldehyde with a hydroxyl group, as in salicylaldehyde, would favor a more stable Schiff base and could thus increase the protective effect as compared with benzaldehyde. The experiments are aimed at determining if there is a correlation between the formation of Schiff bases by these benzaldehyde derivatives and, when combined simultaneously, their ability to protect against *cis*-DDP-induced cytotoxicity and platinum accumulation.

MATERIALS AND METHODS

Cell line and cell synchronization. Cells of the human line NHIK 3025, established from a cervical carcinoma *in situ* were cultivated as monolayers in medium E2a supplemented with 20% human and 10% horse serum as described previously [6, 7]. The cells were recultured every second or third day to ensure continuous, exponential growth. Synchronized cells were obtained by shake-off of mitotic cells [8]. In all experiments utilizing synchronized cells the entire experimental procedure took place in a 37° walk-in incubator room. Under growth conditions as used here, the NHIK 3025 cells normally

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† Abbreviations used: *cis*-DDP, *cis*-dichlorodiammineplatinum; BG, 4,6-benzylidene-D-glucose.

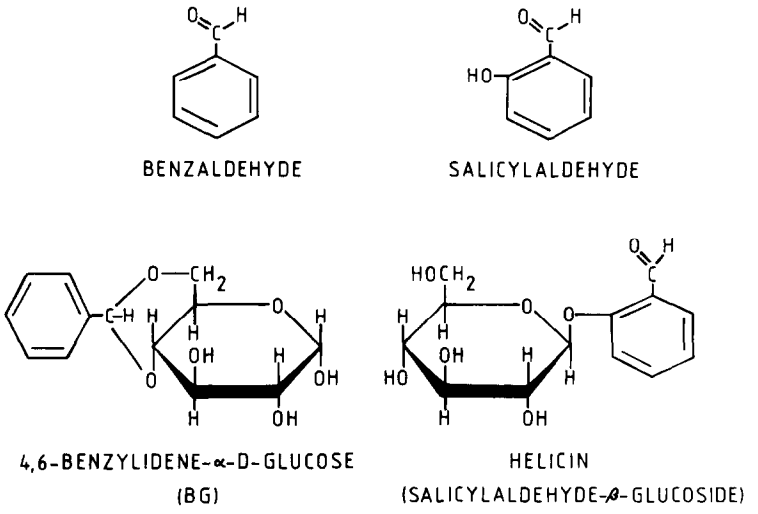

Fig. 1. Chemical structure of benzaldehyde, 4,6-benzylidene-D-glucose (BG), salicylaldehyde and salicylaldehyde-β-glucoside (helicin). For BG the α-isomer is shown, although the compound exists as both α- and β-isomers when in aqueous solution.

have a median cell-cycle duration of ~18 hr, with median G1, S and G2 durations of ~6.5, ~8, and ~2.5 hr, respectively. Duration of mitosis was about 50 min.

Cell survival. As the selected mitoses existed in suspension immediately after shake-off, controlled volumes were seeded directly into plastic tissue culture flask (Falcon 3013, 25 cm^2; Falcon Plastics, Oxnard, CA, U.S.A.). Within 2 hr all the mitotic cells completed division and attached to the bottom of the flasks as doublets. Cell survivial in experiments using synchronized cells was thus corrected for a cell multiplicity of two in order to represent the survival of single cells. For experiments involving asynchronous cells, the cells were trypsinized and a known number were seeded into 60 × 15 mm style (21 cm^2) plastic Petri dishes (Falcon 3002). Dishes were kept in a CO_2 incubator holding 37°, while flasks were stored in the 37° incubator room after flushing with a mixture of 5% CO_2 in air.

Drugs were added to the exponentially growing, asynchronous cells 2 hr after the cells were seeded by replacing the medium in the dishes with medium containing the desired concentrations of the drugs. Drugs were added to the synchronized cells at various times after seeding, but not earlier than 2 hr after selection. Following the drug treatment period, the dishes or flasks were rinsed with warm (37°) Hanks' balanced salt solution before fresh medium was added. Flasks were flushed with 5% CO_2 in air each time they were opened. After 12 to 14 days, colonies of cells were fixed in ethanol and stained with methylene blue. Only colonies containing more than 40 cells were scored as survivors, and data are expressed as the surviving fraction, i.e. the fraction of cells not inactivated by treatment and giving rise to macroscopic colonies relative to control cells.

Cell cycle kinetics. The progression of the synchronized cells through the cell cycle (i.e. the time of entrance into S-phase and the rate of DNA accumulation) was determined from DNA histograms of flow cytometric recordings taken at various times after mitotic selection as previously described [6, 7]. Cell division was recorded in a separate 25-cm^2 flask seeded immediately after cell synchronization where all cells within a delineated area of the flask (~100 cells) were observed repeatedly in an inverted microscope. The time of entrance into mitosis as well as the time of division was noted for each cell within the delineated area.

Atomic absorption spectroscopy. Analysis of cell-associated platinum was performed using a Varian SpectrAA-30 atomic absorption spectrometer fitted with a GTA-96 graphite tube atomizer. Instrument control and data acquisition were by Varian Atomic Absorption Software. Automatic background correction with a modulated deuterium lamp was utilized. Cells were loosened from flasks by trypsin treatment and counted. Cells (2 × 10^6) were added to conical centrifuge tubes, three replicate tubes for each drug concentration. The tubes were centrifuged and cells were resuspended in drug-containing medium, usually 3 mL/tube. The cells were incubated with drugs at 37° and held in suspension by Using a rotary rack. After treatment, the cells were centrifuged and washed in phosphate-buffered saline (PBS: 8000 mg/L NaCl, 1150 mg/L $Na_2HPO_4 \cdot 2H_2O$, 200 mg/L KH_2PO_4, 200 mg/L KCl). The cell pellet was taken up into 100 μL concentrated HNO_3. Following overnight oxidation of organic material, 100 μL H_2O was then added to each tube. Aliquots of 25 μL (representing 250,000 cells) were then placed in a graphite tube and the atomic absorption signal measured at 265.9 nm was registered. Platinum content was quantitated by running a calibration curve immediately before the samples.

Drugs. The chemical structures of the aldehyde and aldehyde-derivatives used in this study are shown in Fig. 1. Benzaldehyde was purchased from Koch-Light Laboratories Ltd (Colnbrook, U.K.) and was vacuum-distilled and stored under N_2. Salicylaldehyde and helicin were products of Aldrich Chemie GmbH (Steinheim, F.R.G.). 4.6-Benzylidene-D-glucose (BG) was generously supplied by

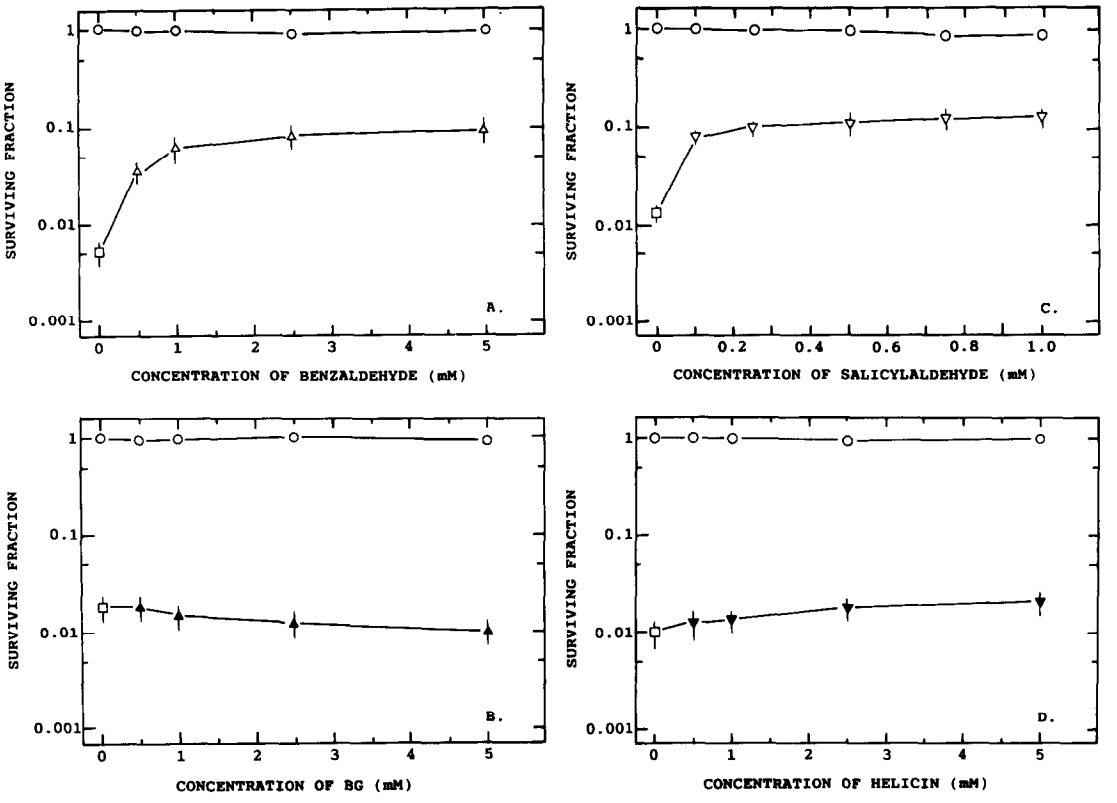

Fig. 2. Surviving fraction of asynchronous NHIK 3025 cells as a function of the concentration of aldehydes or aldehyde derivatives alone or in simultaneous combination with *cis*-DDP. Single cells were plated in plastic Petri dishes 2 hr before medium with the appropriate concentration of drugs was added. Cells were then treated for 2 hr with 10 μM *cis*-DDP alone (□) or in simultaneous combination with (A) benzaldehyde (△) (redrawn from Ref. 1), (B) BG (▲), (C) salicylaldehyde (▽), or (D) helicin (▼). The surviving fraction of cells treated for 2 hr with each aldehyde or aldehyde derivative alone is indicated by (○). Thereafter, the dishes were rinsed with Hanks' balanced salt solution then fresh medium was added. Colonies of cells were fixed and stained 12–14 days after the start of the experiment. The experimental points represent the mean of at least two independent experiments each consisting of five dishes per drug concentration. SE is represented by vertical bars when exceeding the size of the symbols.

Dr Mutsuyuki Kochi (Ichijo-kai Hospital, Chiba, Japan). We have previously shown [9] that BG contains <0.1% free benzaldehyde and when in aqueous solution two isomers, benzylidene-α- and benzylidene-β-glucose, are present at a ratio of 37% and 63% respectively. *cis*-Dichlorodiammineplatinum (Platistin) was from Farmitalia Carlo Erba (Barcelona, Spain). Stock solutions of aldehydes and aldehyde derivatives were made up in medium E2a. *cis*-DDP was dissolved in Hanks' balanced salt solution. All solutions were sterilized by membrane filtration. Drug dilutions and drug combinations were made in medium E2a immediately before use.

RESULTS

The combined effect of aldehydes and aldehyde derivatives on *cis*-DDP-induced cell inactivation was tested by treating NHIK 3025 cells with each compound either singly or in combination with *cis*-DDP. Results from these experiments are shown in Fig. 2 where the fraction of cells surviving treatment and giving rise to macroscopic colonies is plotted against the aldehyde concentration used. In panel A of Fig. 2 the results obtained following a 2 hr treatment of cells with benzaldehyde either alone or in simultaneous combination with 10 μM *cis*-DDP are shown. While benzaldehyde alone up to 5 mM had little effect on cell survival, the inactivating effect of *cis*-DDP was reduced considerably by the simultaneous presence of benzaldehyde at concentrations above 0.5 mM as indicated by an increase in the surviving fraction. Optimal protection occurred when treatment included 1 mM or higher concentrations of benzaldehyde. Treatment of cells with the benzaldehyde-glucose acetal BG did not, however, have any effect on *cis*-DDP-induced cell inactivation (panel B). Panel C demonstrates that salicylaldehyde induces a protection against *cis*-DDP-induced cell inactivation, and comparison with panel A indicates that this effect is of about the same magnitude as that induced by benzaldehyde. However, for salicylaldehyde optimal protection was achieved using concentrations as low as 0.2 mM. The salicylaldehyde-glucoside helicin also reduced *cis*-DDP-induced cell inactivation, but only very modestly (panel D). Maximum protection was seen when cells were treated with 5 mM helicin in combination

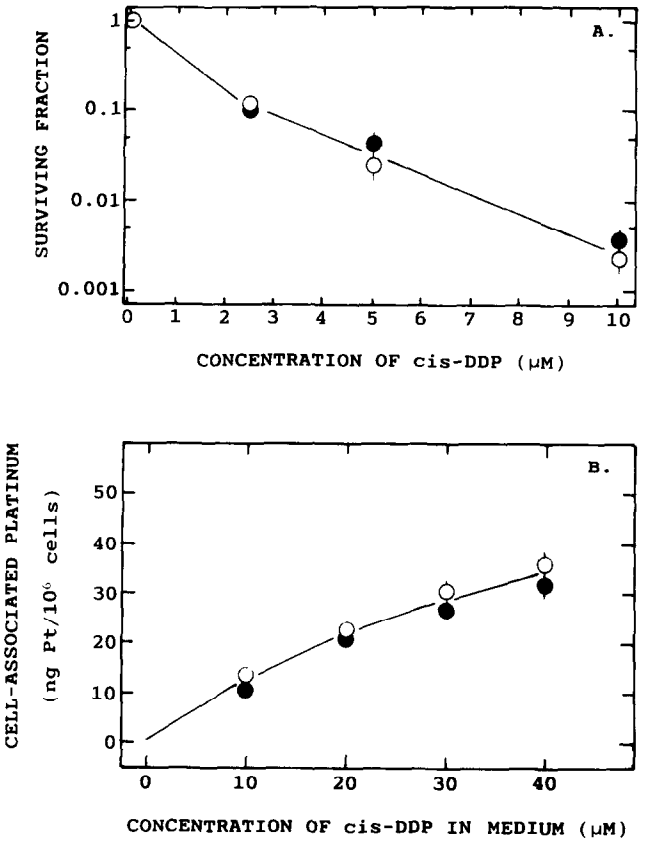

Fig. 3. (A) Surviving fraction of asynchronous NHIK 3025 cells as a function of the concentration of *cis*-DDP. Cells were treated either attached to plastic dishes as monolayers (○), or in suspension (●) with various concentrations of *cis*-DDP for 2 hr. Thereafter, dishes were rinsed with warm (37°) Hanks' balanced salt solution then fresh medium was added. Known numbers of cells in suspension were treated with *cis*-DDP, then centrifuged in conical centrifuge tubes. Cells were washed in warm Hanks' balanced salt solution and resuspended in warm medium E2a. Known numbers of cells were then seeded into plastic Petri dishes and incubated for colony formation. Each point represents the mean colony count from five replicate dishes, vertical bar represent SE. (B) Cell-associated platinum content of NHIK 3025 cells as a function of the concentration of *cis*-DDP in medium. Cells were treated with *cis*-DDP either in suspension or attached to plastic dishes as monolayers. Following treatment, suspension cells were washed with PBS and resuspended in NHO_3. Monolayer cells were loosened by trypsin treatment and counted. Aliquots of cells representing similar numbers as for suspension cultures were washed with PBS and resuspended in HNO_3. The platinum content was determined as described in Materials and Methods.

with *cis*-DDP, this concentration being the highest studied here.

As we have previously found that benzaldehyde also reduces cellular accumulation of *cis*-DDP [2], we set up experiments in which the amount of cell-associated platinum was measured by flameless atomic absorption spectroscopy. As described in Materials and Methods, the use of cells in suspension simplified platinum atomic absorption spectroscopy measurements. Therefore, we first had to insure that both the cell survival following treatment with *cis*-DDP and measurements of cell-associated platinum would not be affected by the culture conditions used, i.e. monolayer or suspension. We had previously found that survival was not affected to any great degree when cells were treated with *cis*-DDP either attached to plastic dishes [1] or in suspension [2]. The data shown in Fig. 3 (panel A) reconfirms these observations. In addition, the processes involved in the accumulation of platinum within the cell, at least for a steady-state condition, also do not seem to be affected by culture conditions (Fig. 3, panel B).

In Fig. 4, the amount of platinum associated with cells (ng $Pt/10^6$ cells) following a 2 hr treatment period with 30 μM *cis*-DDP either alone, or in simultaneous combination with increasing benzaldehyde derivative concentrations is shown. The concentration of *cis*-DPP (30 μM) was chosen in order to yield a platinum atomic absorption signal of sufficient quantitative intensity. In panel A, cells treated simultaneously with *cis*-DDP are shown to contain less platinum when the combination includes benzaldehyde, but not BG. Optimum reduction in platinum accumulation occurred at benzaldehyde concentration of from 1 mM. Even at 5 mM BG the amount of cell-associated platinum was within the standard error of the platinum quantitation from cells treated with *cis*-DDP alone. In panel B of Fig.

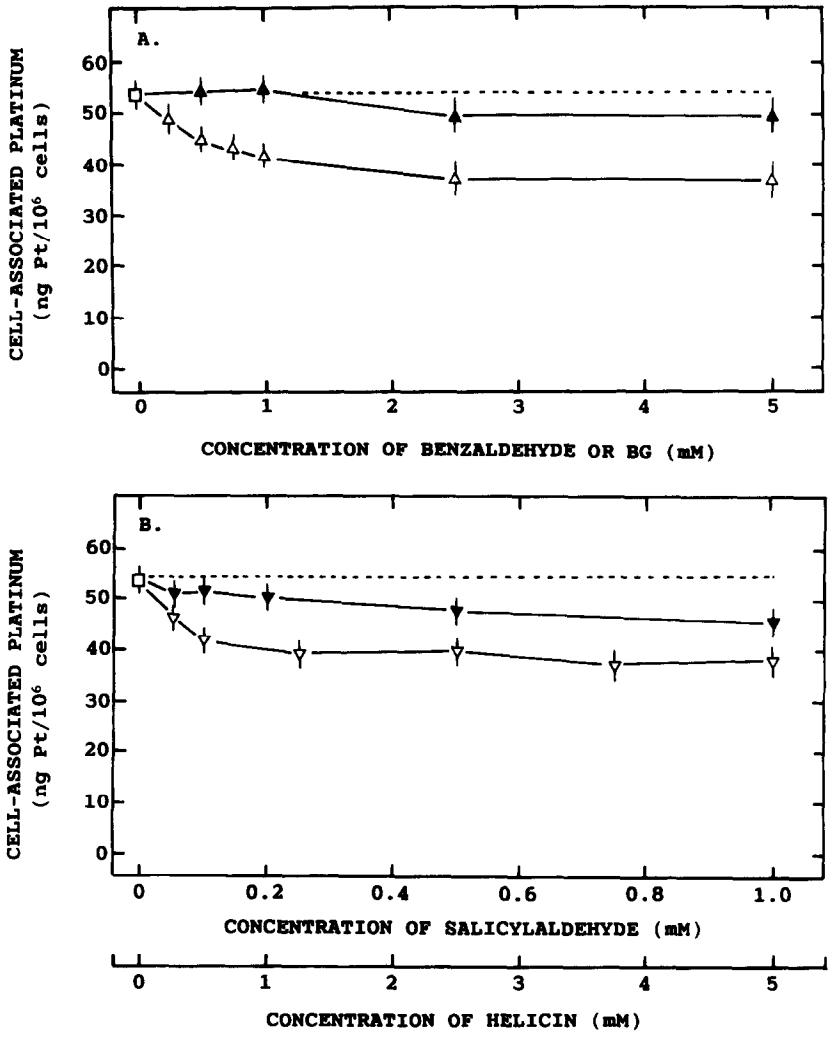

Fig. 4. Cell-associated platinum content of NHIK 3025 cells as a function of the concentration of (A) benzaldehyde (△) or BG (▲); (B) salicylaldehyde (▽) or helicin (▼) in simultaneous combination with 30 μM *cis*-DDP. Cell-associated platinum content of cells treated with *cis*-DDP alone is indicated by (□). Asynchronous NHIK 3025 cells were trypsinized and resuspended in drug-containing medium, 2×10^6 cells in 3 mL. Incubation was for 2 hr at 37° with gentle but continuous rotation of the samples. Cells were then centrifuged, washed with PBS, and finally resuspended in 100 μL concentrated HNO_3. After overnight oxidation of organic material, 100 μL H_2O was added to each sample and cell-associated platinum was determined by flameless atomic absorption spectoscopy. Each experimental point represents the mean of six platinum determinations, and vertical bars represent SE.

4 cells were treated simultaneously with *cis*-DDP and either salicylaldehyde or helicin. Both aldehyde compounds induce a reduction in the amount of cell-associated platinum, with salicylaldehyde treatment being more effective than helicin both on a molar basis and with respect to the degree of reduction. The above described experiments demonstrate that benzaldehyde, salicyladehyde and to some degree helicin, but not BG, protect cells from *cis*-DDP-induced cell inactivation by decreasing cellular accumulation of platinum.

In order to distinguish between platinum accumulation and short-term uptake, we determined the cell-asociated platinum content of NHIK 3025 cells at various times during a 2 hr incubation period. The data in Fig. 5 show that the intracellular platinum content increases with increasing exposure of cells to platinum-containing medium. For incubation periods of 15 min and longer the simultaneous presence of benzaldehyde induced a reduction in platinum accumulation. This reduction mimicked a lengthening of incubation times, that is, the incubation time necessary to yield a given cell-associated platinum content (for example 20 ng Pt/10^6 cells) was twice as long for cells simultaneously exposed to benzaldehyde (34 min) than cells exposed to *cis*-DDP alone (18 min).

The next question we were concerned with was whether or not the protective effect of aldehydes was dependent upon the position of cells in the cell cycle. To answer this question we treated synchronized NHIK 3025 cells with 2 hr drug pulses at various times following mitotic selection. Figure 6 shows the results for such an experiment expressed as the

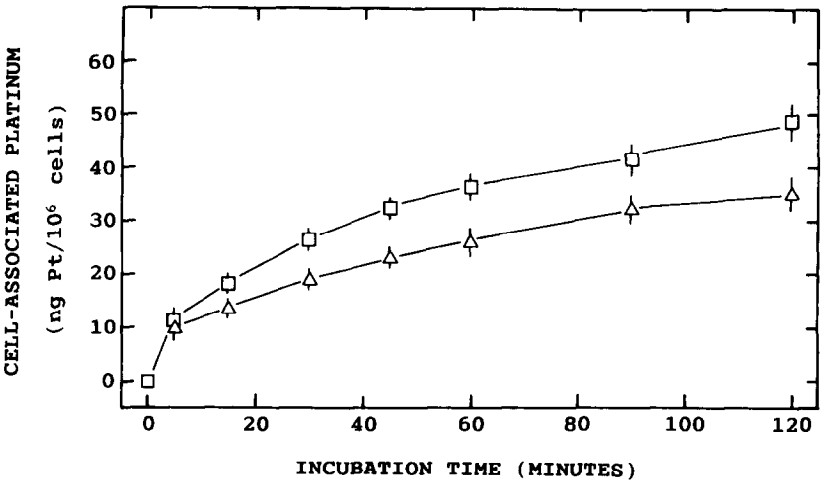

Fig. 5. Cell-associated platinum content of NHIK 3025 cells as a function of the incubation times in the presence of 30 μM *cis*-DDP alone (□) or in simultaneous combination with 2.5 mM benzaldehyde (△). Cells were treated in suspension, aliquots removed at various times, and the platinum content determined as described in Materials and Methods.

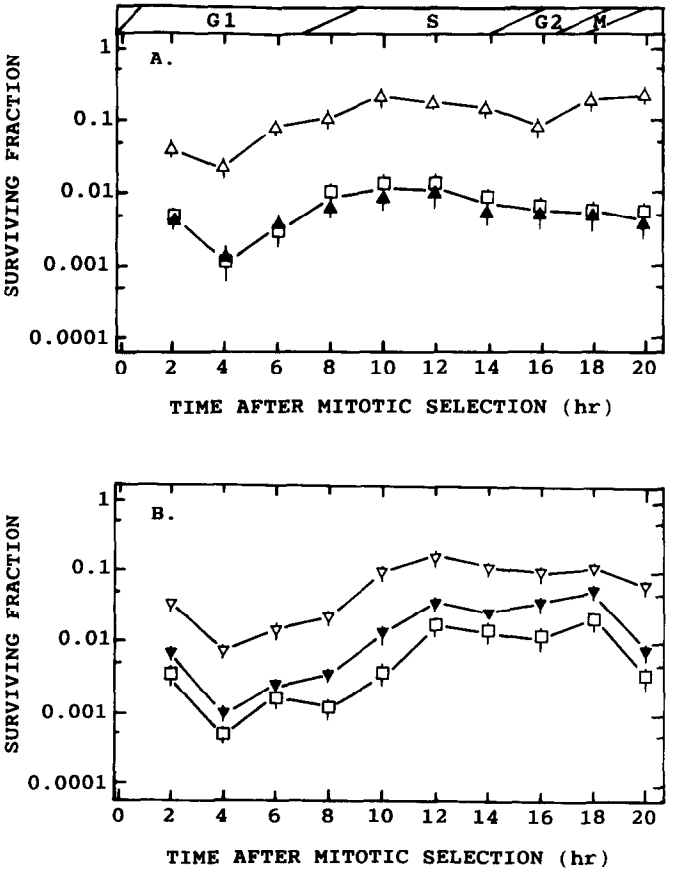

Fig. 6. Surviving fraction of synchronized NHIK 3025 cells treated for 2 hr with 10 μM *cis*-DDP alone (□) or in simultaneous combination with (A) 2.5 mM benzaldehyde (△) or 5 mM BG (▲); (B) 0.25 mM salicylaldehyde (▽) or 5 mM helicin (▼) as a function of the time after mitotic selection. Experimental points from one experiment are plotted from the time at which drug incubation began. The duration of the various cell-cycle phases for control cells is shown at the top of panel A. Vertical bars represent SE.

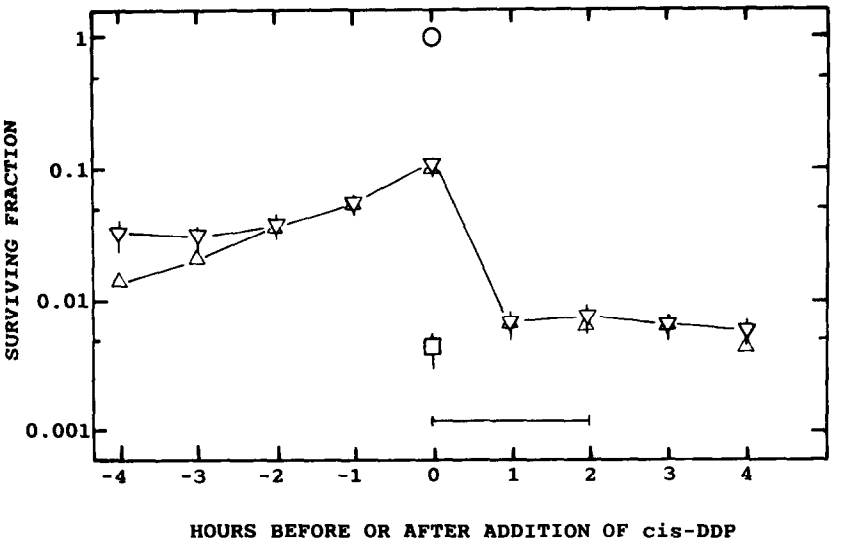

Fig. 7. Surviving fraction of asynchronous NHIK 3025 cells treated with 10 μM *cis*-DDP as a function of scheduling of 2-hr pulses of 0.25 mM salicylaldehyde (▽). (○) and (□) represent cell survival after a 2-hr pulse of 0.25 mM salicylaldehyde alone or 10 μM *cis*-DDP alone, respectively. The horizontal bar represents the treatment period for *cis*-DDP and data points are plotted at the time at which drug incubation began. (△) represents experimental data for 3.2 mM benzaldehyde redrawn from Ref. 1. Each experimental point represents the mean colony count from five replicate flasks. Vertical bars represent SE.

fraction of cells surviving treatment versus the time after mitotic selection at which drug treatment began. From the previous experiments and to ensure that all aldehyde binding sites could be occupied, we chose the following concentrations for further study: 2.5 mM benzaldehyde, 5 mM BG, 0.25 mM salicylaldehyde and 5 mM helicin. Treatment of synchronized cells with aldehydes or aldehyde derivatives alone did not induce any significant cell inactivation through the cell cycle at the concentrations utilized. While the fraction of cells surviving treatment with 10 μM *cis*-DDP is lower in G1-phase than in other cell cycle phases, the simultaneous presence of 2.5 mM benzaldehyde increased the fraction of surviving cells in all phases of the cell cycle. The inability of BG to protect cells from inactivation by *cis*-DDP is also illustrated clearly in panel A. In addition, salicylaldehyde also protected cells from inactivation by *cis*-DDP throughout the cell cycle (panel B). This protective effect was of about the same magnitude as that seen for benzaldehyde, although treatment was with only 0.25 mM salicylaldehyde as opposed to 2.5 mM benzaldehyde. Helicin (5 mM) also increased the fraction of cells surviving a 10 μM *cis*-DDP treatment, although only slightly in comparison to salicylaldehyde or benzaldehyde.

In experiments described thus far, *cis*-DDP and aldehydes were added and removed simultaneously for combined treatment. We have also studied the combined effects where the treatment period for the two drugs were separate or overlapping only partially. The data in Fig. 7 represent such an experiment where 10 μM *cis*-DDP was present for 2 hr (horizontal line marking from 0 to 2 hr) and salicylaldehyde (inverted triangles) was present as 2-hr pulses either before, during, or after the *cis*-DDP treatment period. Survival is plotted as a function of the time when salicylaldehyde was added. Cells treated with 10 μM *cis*-DDP alone (square) or 0.25 mM salicylaldehyde alone (circle) are also shown. Furthermore, for comparison, data for treatment with 3.2 mM benzaldehyde (triangles) is redrawn from Ref. 1.

From Fig. 7, salicylaldehyde (0.25 mM) given simultaneously with 10 μM *cis*-DDP resulted in an increase in cell survival from that following treatment with *cis*-DDP alone. Salicylaldehyde (or benzaldehyde) treatment following the *cis*-DDP pulse resulted in early identical survival as with *cis*-DDP alone. When salicylaldehyde (or benzaldehyde) treatment preceded that of *cis*-DDP, significantly greater cell survival than after *cis*-DDP alone was observed. For the two aldehydes, though, differences in the magnitude of the protective effect were observed when aldehyde treatment preceded *cis*-DDP treatment by 4 hr. At this time point, salicylaldehyde induced a greater protective effect than benzaldehyde, although present at only 0.25 mM compared to 3.2 mM benzaldehyde. Neither aldehyde, however, induced maximum protection until simultaneous drug treatment occurred. We believe this difference between salicylaldehyde and benzaldehyde may be due to the stability of a Schiff base formed between the aldehyde and a cell membrane amino group, as described below.

From the above described cell inactivation and platinum accumulation experiments, data was obtained from which a correlation between cellular platinum content and survival could be made. In Fig. 8 the fraction of cells surviving treatment with *cis*-DDP or *cis*-DDP + benzaldehyde derivatives is plotted against the cell-associated platinum content of treated cells. The straight line represents a linear

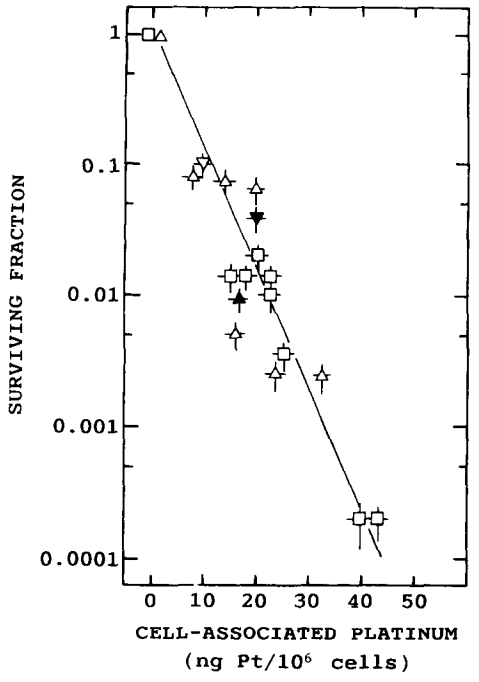

Fig. 8. The fraction of human NHIK 3025 cells surviving treatment with *cis*-DDP or *cis*-DDP + benzaldehyde derivatives versus the cell-associated platinum content. Cells were treated for 2 hr with *cis*-DDP alone (□) or *cis*-DDP in simultaneous combination with 2.5 mM benzaldehyde (△), 5 mM BG (▲), 0.25 mM salicylaldehyde (▽), or 5 mM helicin (▼), then either seeded for colony survival or analysed for platinum content by atomic absorption spectroscopy. Vertical bars represent SE associated with the surviving fraction, horizontal bars refer to SE associated with the platinum content.

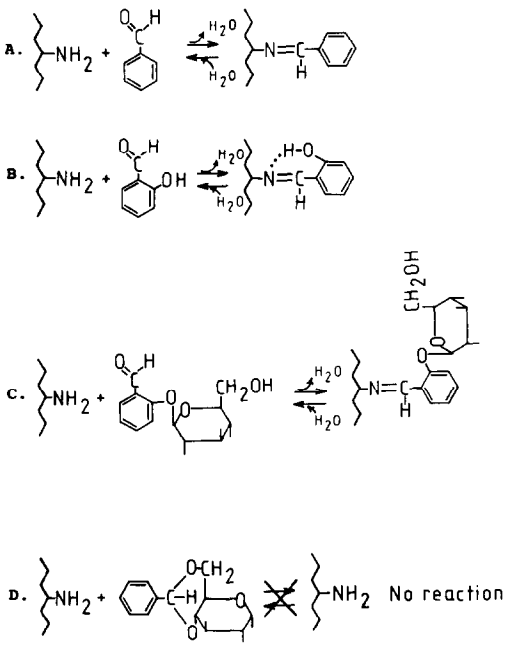

Fig. 9. Proposed formation of a Schiff base imine between cellular amino groups and (A) benzaldehyde, (B) salicylaldehyde, (C) helicin and (D) BG.

regression fit to data from cells treated with *cis*-DDP alone. Data from cells treated with *cis*-DDP + benzaldehyde derivatives appear to fall on, or near, this line ($P = 0.16$). For instance, treatment of cells for 2 hr with 10 μM *cis*-DDP in the culture medium resulted in a platinum content of 22.1 ng Pt/10^6 cells, and a surving fraction of 0.012. Simultaneous presence of 2.5 mM benzaldehyde increased the fraction of cells surviving a 2-hr treatment with 10 μM *cis*-DDP to 0.10, and effectively led to a decrease in the platinum content to 11.4 ng Pt/10^6 cells.

DISCUSSION

Since we propose that a reaction between cell membrane amino groups and aldehydes is of importance for protection against *cis*-DDP-induced cell inactivation [2], then the efficiency of the protection would depend on the nature of the Schiff base formed. Using hemoglobin as a source of amino groups Zaugg *et al.* [10] have shown that aromatic aldehydes have a two- to three-fold increased reactivity over aliphatic aldehydes in Schiff base formation. Means and Feeney [11] have also described reactions between carbonyl compounds and free amino groups of proteins to form reversible Schiff base linkages. These previous reports thus indicate that formation of a Schiff base is an important reaction mechanism when aromatic aldehydes and amino groups are present. In Fig. 9 we show proposed reactions between cell membrane amino groups and the aldehydes or aldehyde derivatives studied.

Benzaldehyde and salicylaldehyde readily form Schiff base imines with membrane amino groups and, in fact, high equilibrium constants have been measured for benzaldehyde reacting with amines [12, 13]. For salicylaldehyde, however, two types of reactions due to the presence of a hydroxyl group at the position adjacent to the aldehyde moiety can occur. Firstly, internal hydrogen bonding between the hydroxyl hydrogen and the carbonyl group would lead to development of a partial positive charge on the carbonyl carbon. This, in turn, would increase reaction kinetics between salicylaldehyde and nucleophilic groups such as amino groups. Secondly, the imine could be stabilized due to hydrogen bonding between the lone-pair electrons of the imine nitrogen and the hydroxyl group hydrogen of salicylaldehyde [11]. This stabilization of Schiff base adducts leads to the same expression of protection against *cis*-DDP-induced cell inactivation as benzaldehyde, but an optimal protective effect is achieved uisng only one-fifth (0.2 mM vs 1 mM) the salicylaldehyde concentration than benzaldehyde (Figs 2 and 4). In addition, the stability of the formed Schiff base could have greater longevity than that formed by benzaldehyde, thus explaining greater protection induced by salicylaldehyde than benzaldehyde when aldehyde treatment preceded *cis*-DDP treatment (Fig. 7).

Helicin, possessing a large glucose residue adjacent to the aldehyde moiety, could be sterically hindered from Schiff based formation [14, 15]. Still, one

may expect some Schiff based formation to occur at cell membrane sites which would allow the approach of the helicin molecule [10].

BG, not possessing a free aldehyde moiety, would not interact at all with membrane amino groups and thus, according to our hypothesis, would not have any effect on either *cis*-DDP-induced cell inactivation or on cellular uptake of *cis*-DDP. The results shown in Figs 2, 4 and 6 confirm this supposition.

The present results concerning the combination of BG with *cis*-DDP allow us to make two additional conclusions. Firstly, since both benzaldehyde and BG are equally effective as protein synthesis inhibitors [9], it does not appear that an inhibition of protein synthesis *per se* is responsible for the protective effect displayed when cells are treated with benzaldehyde and *cis*-DDP in simultaneous combination (Fig. 2) [1]. Secondly, both BG and helicin possess bulky glucose residues, but only helicin protects against *cis*-DDP, indicating the absolute requirement for a reactive aldehyde moiety.

The results from this report and our previous work allow us to speculate on the nature of *cis*-DDP uptake into cells. Benzaldehyde, salicylaldehyde, and helicin reduce the accumulation of *cis*-DDP into cells (Figs 4 and 5) either by sterically hindering *cis*-DDP or by blocking a membrane-specific platinum uptake site via Schiff base formation with cell membrane amino groups [2]. The biological result of reduced platinum accumulation is protection from *cis*-DDP-induced cell inactivation (Fig 2, 6 and 7), and a correlation between platinum content on the one hand and cell survival on the other hand appears to be valid (Fig. 8). However, even with salicylaldehyde which was the most potent aldehyde tested, the protective effect cannot be increased beyond a certain limit. This limit is clearly shown by a plateau appearing in both cell inactivation (cf. Fig. 2) and platinum accumulation studies (Fig. 4).

The reduction in platinum accumulation was not immediate, however, indicating either that aldehyde binding to the cell membrane requires a certain amount of time (at least 5 min), or that there is a requirement for a minimum amount of aldehyde to be bound to membrane sites specific for *cis*-DDP uptake before changes in platinum accumulation occur (Fig. 5). Both benzaldehyde and salicylaldehyde protect cells from *cis*-DDP-induced cell inactivation throughout the cell cycle (Fig. 6), indicating that the binding sites (or amino groups involved in Schiff base formation) for benzaldehyde and salicylaldehyde do not appear to be altered relative to cell age.

While it is possible that Schiff base formation could lead to non-specific changes in membrane structure, which in turn could slow *cis*-DDP diffusion into cells, this effect can not be large. We have previously shown that benzaldehyde does not affect the cell inactivating effect induced by the two alkylating agents 1,3-bis(2-chloroethyl)-1-nitrosourea and nitrogen mustard [1]. Furthermore, aliphatic aldehydes do not induce any protective effect when in combination with *cis*-DDP [1, 16]. Therefore a prerequisite for induction of a protective effect against *cis*-DDP cytotoxicity is not only the presence of an aldehyde moiety, but the aldehyde must also possess aromatic character.

Aromatic aldehydes could also compete for intracellular *cis*-DDP binding sites, or more appropriately, alter the secondary structure of DNA in such a manner that *cis*-DDP cannot bind to DNA [17]. Alternately, the efflux of platinum from the cell could be affected by benzaldehyde treatment. Preliminary investigations have not shown there to be measurable efflux of platinum from NHIK 3025 cells within the 2 hr treatment period, nor any increase in platinum efflux induced by benzaldehyde (JE Melvik, personal communication). The protective effect due to benzaldehyde derivatives may thus represent blockage of "active" or membrane-mediated platinum uptake sites, while passive diffusion of *cis*-DDP into cells is not affected by Schiff base formation. Such membrane sites for platinum uptake have been suggested by Byfield and Calabro-Jones [18] and by Scanlon *et al.* [19] which could be indistinguishable from amino acid carriers. Just recently a possible energy dependent pathway for platinum uptake has been suggested by Andrews *et al.* [20]. We cannot say at present if aldehyde protection also involves a disruption of energy equilibria within cells, but a previous report [9] in which the vitamin B_6 aldehyde pyridoxal 5'-phosphate (a membrane impermeable aldehyde) induced a protective effect indicates that the outside of the cell membrane may possess special sites for platinum uptake.

Acknowledgements—This study was supported by the Norwegian Cancer Society. The expert technical assistance of Charlotte Borka and Ursula Prehn Hansen is appreciated. The Nansen Fund provided financial support for the Perkin-Elmer Model 3600 Data Station used for cell survival analysis. The Norwegian Research Council for Science and the Humanities (NAVF) provided funds for the Varian SpectrAA-30 atomic absorption spectrometer.

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