Synergistic Cell Inactivation of Human NHIK 3025 Cells by Cinnamaldehyde in Combination with cis-Diamminedichloroplatinum(II)\(^1\)

John M. Dornish,\(^2\) Erik O. Pettersen, and Reidar Oftebro
Department of Tissue Culture, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 Oslo 3, Norway

**ABSTRACT**

The cell-inactivating effect induced by cinnamaldehyde in combination with cis-diamminedichloroplatinum(II) (cis-DDP) on human NHIK 3025 cells in culture was investigated. Cell inactivation was measured as a loss in the ability of single cells to give rise to macroscopic colonies following drug treatment. Although 2 h treatment of asynchronous cells with 0.3 mm cinnamaldehyde alone induced little cell inactivation, the drug combination of 0.3 mm cinnamaldehyde and 10 \(\mu\)M cis-DDP resulted in synergistic cell inactivation. Cinnamaldehyde potentiated the cell-inactivating effect of cis-DDP by a dose-modifying factor of 1.8. Drug synergism was found to occur only when cinnamaldehyde and cis-DDP were given in simultaneous combination. Treatment of synchronized cells demonstrated that cinnamaldehyde potentiated the inactivating effect of cis-DDP in all phases of the cell cycle. Cinnamic acid and cinnamyl alcohol were found to have no synergistic or potentiating effect on cell survival following treatment of cells with cis-DDP, thus indicating the importance of an aldehyde moiety for drug synergism.

**INTRODUCTION**

Cinnamaldehyde (trans-3-phenylpropenal) has been shown to possess antifungal activity (1–3). This aldehyde has also been reported to inhibit growth and protein synthesis of L1210 cells (4). We have previously shown that the simplest aromatic aldehyde, benzaldehyde, and its glucose-acetal derivative, 4,6-benzylidene-d-glucose also inhibit protein synthesis in cultured human cells (5–7). In addition, benzaldehyde as well as the vitamin B\(_6\) aldehydes pyridoxal and pyridoxal 5'-phosphate have been found to protect cells from the inactivating effects of cis-DDP\(^1\) (8, 9). It appears that benzaldehyde inhibits cellular uptake of cis-DDP by forming a Schiff base with cell membrane amino groups (9, 10). Since cinnamaldehyde also forms Schiff's bases with proteins, although to a lesser extent than benzaldehyde (11), we wanted to investigate the effect cinnamaldehyde would have on cis-DDP-induced cell inactivation.

**MATERIALS AND METHODS**

Cell Line and Cell Synchronization. Cells of the human line NHIK 3025, established from a cervical carcinoma in situ (12, 13), were cultivated as monolayers in medium E2a (14) supplemented with 20% human (prepared at the laboratory) and 10% horse serum (Grand Island Biological Co., Renfrewshire, Scotland). The cells were recultured every second or third day to ensure continuous, exponential growth. Synchronization was found to occur only when cinnamaldehyde and cis-DDP were given in simultaneous combination. Treatment of asynchronous cells demonstrated that cinnamaldehyde potentiated the inactivating effect of cis-DDP in all phases of the cell cycle. Cinnamic acid and cinnamyl alcohol were found to have no synergistic or potentiating effect on cell survival following treatment of cells with cis-DDP, thus indicating the importance of an aldehyde moiety for drug synergism.

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\(^2\)Fellow of the Norwegian Cancer Society. To whom requests for reprints should be addressed.

\(^3\)The abbreviation used is: cis-DDP, cis-diamminedichloroplatinum(II).
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Fig. 1. The chemical structure of cinnamaldehyde.

Fig. 2. The surviving fraction of NHIK 3025 cells as a function of the concentration of cinnamaldehyde (○), cinnamaldehyde + 5 μM cis-DDP (△) or cinnamaldehyde + 10 μM cis-DDP (▽). Cells were treated with drugs or drug combinations for 2 h, then washed with Hanks’ balanced salt solution. Points, mean colony count from two independent experiments each consisting of five replicate dishes per point; bars, SE.

RESULTS

To test aldehyde-mediated modulation of cis-DDP-induced cell inactivation, we treated NHIK 3025 cells simultaneously with both cinnamaldehyde and cis-DDP. Fig. 2 shows the results of experiments in which cells were treated for 2 h with varying concentrations of cinnamaldehyde up to 0.5 mM either alone or in combination with 5 μM or 10 μM cis-DDP. Cinnamaldehyde treatment alone induced little cell inactivation at concentrations below 0.5 mM. However, the simultaneous combination with cis-DDP produced synergistic inactivating effects. With 0.5 mM cinnamaldehyde and 5 μM cis-DDP similar surviving fractions of cells were found as with 10 μM cis-DDP alone. With a higher cis-DDP concentration (10 μM), the potentiation was even stronger.

The importance of the aldehyde moiety for potentiation of cis-DDP-induced cell inactivation by cinnamaldehyde is shown by the data in Table 1. Neither cinnamic acid nor cinnamyl alcohol had any synergistic (or antagonistic) effect when tested in combination with cis-DDP.

<table>
<thead>
<tr>
<th>Drug treatment (2 h)</th>
<th>Single treatment (no cis-DDP)</th>
<th>Combination with 10 μM cis-DDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>0.018 ± 0.002</td>
</tr>
<tr>
<td>0.3 mM cinnamic acid</td>
<td>0.92 ± 0.07</td>
<td>0.022 ± 0.001</td>
</tr>
<tr>
<td>0.3 mM cinnamaldehyde</td>
<td>0.94 ± 0.06</td>
<td>0.0018 ± 0.0002</td>
</tr>
<tr>
<td>0.3 mM cinnamyl alcohol</td>
<td>0.95 ± 0.06</td>
<td>0.021 ± 0.001</td>
</tr>
</tbody>
</table>

Fig. 3 shows the surviving fraction of asynchronous NHIK 3025 cells following a 2-h treatment with either cis-DDP alone or cis-DDP in combination with 0.3 mM cinnamaldehyde as a function of the concentration of cis-DDP. The data indicate that cinnamaldehyde potentiates the cell inactivating effect of cis-DDP by a dose-modifying factor of approximately 1.8, i.e., the effect of 10 μM cis-DDP in combination with 0.3 mM cinnamaldehyde is similar to that of 18 μM cis-DDP alone. This is demonstrated more clearly in Table 2 where D0 values for each segment of the biphasic cell inactivation curves for cis-DDP and cis-DDP + cinnamaldehyde treatment are shown. As can be seen, the dose-modifying factor, calculated as the ratio between D0 values after treatment with either cis-DDP alone or cis-DDP + cinnamaldehyde treatment, is approximately 1.8 irrespective of which part of the cell-inactivation curve is used for calculation.

The ability of cinnamaldehyde to potentiate cis-DDP cell inactivation at different drug treatment times is presented in Fig. 4. In these experiments, NHIK 3205 cells were exposed continuously to 10 μM cis-DDP either alone or in combination with 0.3 mM cinnamaldehyde for up to 4 h. Cinnamaldehyde
Table 2: $D_0$ values and dose-modifying factors derived from data presented in Fig. 3

NHIK 3025 cells were treated for 2 h with varying concentrations of cis-DDP either alone or in simultaneous combination with 0.3 mM cinnamaldehyde. Cell survival (shown in Fig. 3) was plotted as a function of the fraction of cells surviving treatment versus the concentration of cis-DDP. Since the survival curves appear biphasic, $D_0$ values were calculated from linear regression analysis of data points for curve segments representing surviving fractions greater than 0.1, and for curve segments representing surviving fractions less than 0.1.

<table>
<thead>
<tr>
<th>Surviving fraction</th>
<th>cis-DDP alone</th>
<th>cis-DDP + cinnamaldehyde</th>
<th>Dose-modifying factor*</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥0.1</td>
<td>2.29 μM</td>
<td>1.27 μM</td>
<td>1.80</td>
</tr>
<tr>
<td>≤0.1</td>
<td>4.48 μM</td>
<td>2.38 μM</td>
<td>1.88</td>
</tr>
</tbody>
</table>

* Dose-modifying factor calculated as:

$$\frac{D_0 \text{ (cis-DDP)}}{D_0 \text{ (cis-DDP + cinnamaldehyde)}}$$

Fig. 4. Surviving fraction of NHIK 3025 cells as a function of the duration of drug treatment with 0.3 mM cinnamaldehyde (O), 10 μM cis-DDP ( ), or 0.3 mM cinnamaldehyde + 10 μM cis-DDP ( ). Single cells attached to plastic dishes were treated with each drug or drug combination for the time indicated in the figure. Drug treatment was terminated by removal of the drug-containing medium, and thereafter the cells were washed in Hanks’ balanced salt solution and reincubated in fresh medium for colony formation. Two independent experiments, each consisting of five replicate dishes per point, were averaged. Bars, SE.

Treatment alone resulted in almost 100% survival at all treatment times, although a small effect occurred after 4 h treatment with 0.3 mM. As can be seen, the simultaneous presence of 10 μM cis-DDP and 0.3 mM cinnamaldehyde induced a stronger inactivating effect than cis-DDP alone, irrespective of treatment times up to 4 h.

Experiments thus far described were designed for simultaneous drug combinations. We have also examined the combined effects of cinnamaldehyde and cis-DDP where the treatment periods for the two drugs were separated or overlapped only partially. The data in Fig. 5 represent experiments where cells were treated for 2 h with 10 μM cis-DDP (horizontal line marking from 0 to 2 h) while 0.3 mM cinnamaldehyde was present as a 2-h pulse either before, during, or after the cis-DDP treatment period. Survival is plotted as a function of the time when cinnamaldehyde was added. Thus, each experimental point in Fig. 5 is plotted at the time of the start of treatment. Cells treated with 0.3 mM cinnamaldehyde alone or 10 μM cis-DDP alone are also shown.

From Fig. 5, treatment of cells with cinnamaldehyde (0.3 mM) simultaneously with 10 μM cis-DDP resulted in a cell survival one-tenth of that following treatment with cis-DDP alone. Treatment of cells with cinnamaldehyde 2 h before the cis-DDP pulse (i.e., cinnamaldehyde was thus removed just before cis-DDP was added) caused no potentiation in cell inactivation by cis-DDP. The same was true when cinnamaldehyde treatment started immediately after removal of cis-DDP. Partial overlapping of the treatment periods for the two drugs resulted in some synergistic effect, although maximum synergism (and lowest cell survival) occurred only when cinnamaldehyde and cis-DDP were present simultaneously for the entire treatment period.

To determine whether the potentiation of cis-DDP-induced cell inactivation by cinnamaldehyde was specific to any particular phase of the cell cycle, we treated synchronized cell populations with 2-h pulses of 10 μM cis-DDP alone or in combination with 0.3 mM cinnamaldehyde at various times after mitotic selection. The results are shown in Fig. 6. From the data, treatment of synchronized cells with cinnamaldehyde alone (0.3 mM) induced little cell inactivation. Inactivation of synchronized cells by 10 μM cis-DDP alone occurred throughout the cell cycle with G1-phase cells displaying greater sensitivity while mid-S-phase cells displayed less sensitivity to cis-DDP. The drug combination of 0.3 mM cinnamaldehyde and 10 μM cis-DDP inactivated cells in all phases of the cell cycle, and in particular the mid-S-phase resistance to cis-DDP alone was abolished.

Since the cell survival experiments indicated that treatment of cells with cis-DDP in combination with cinnamaldehyde mimicked the survival obtained when cells were treated with almost double the concentration of cis-DDP alone, it was of interest to quantitate the platinum content in treated cells. Measurements of cell-associated platinum as performed by use of flameless atomic absorption spectroscopy are shown in Fig. 7. The amount of cell-associated platinum in asynchronous cells treated for 2 h with cis-DDP alone increased as the concentration of cis-DDP in cell culture medium increased. The simultaneous presence of 0.3 mM cinnamaldehyde did not, however, affect the amount of cell-associated platinum meas-

- Time when cinnamaldehyde was added.
- Each experimental point in Fig. 5 plotted at the time of the start of treatment.
- Cells treated with 0.3 mM cinnamaldehyde alone or 10 μM cis-DDP alone shown.
- Potentiation of cis-DDP-induced cell inactivation by cinnamaldehyde specific.
- Synchronized cell populations treated with 2-h pulses of 10 μM cis-DDP alone or combination.
- Synchronized cells with cinnamaldehyde alone (0.3 mM) induced minimal inactivation.
- Inactivation of synchronized cells by 10 μM cis-DDP alone occurred through the cell cycle.
- Drug combination of 0.3 mM cinnamaldehyde and 10 μM cis-DDP inactivated cells in all phases.
- Mid-S-phase resistance to cis-DDP alone abolished.
- Amount of cell-associated platinum measured.
- Flameless atomic absorption spectroscopy used.
- Amount of cell-associated platinum increased as concentration of cis-DDP increased.
- Simultaneous presence of cinnamaldehyde did not affect.

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**CINNAMALDEHYDE + cis-DDP SYNERGISM**

Fig. 5. Surviving fraction of NHIK 3025 cells treated with 10 μM cis-DDP as a function of scheduling of 2-h pulses of 0.3 mM cinnamaldehyde ( ). O and , cell survival after a 2-h treatment with 0.3 mM cinnamaldehyde alone or 10 μM cis-DDP alone, respectively. Horizontal bar, treatment period for cis-DDP and data points are plotted from the time at which drug incubation began. Points, mean of three independent experiments (five replicate dishes per point). Vertical bars, SE.
CINNAMALDEHYDE + cis-DDP SYNERGISM

Fig. 6. Surviving fraction of synchronized NHK 3025 cells treated for 2 h with 0.3 mM cinnamaldehyde (C), 10 μM cis-DDP (E), or 0.3 mM cinnamaldehyde + 10 μM cis-DDP (A) as a function of the time after mitotic selection. Points, mean of two experiments, each with four replicate flasks per point, and are plotted from the time at which drug incubation was begun. The duration of the various cell-cycle phases for control cells is shown at the top of the figure. Vertical bars, SE.

Fig. 7. Cell-associated platinum content measured by flameless atomic absorption spectroscopy of NHK 3025 cells treated for 2 h with cis-DDP either alone (C) or in simultaneous combination with 0.3 mM cinnamaldehyde (Δ). Cells were treated in suspension, then washed in phosphate-buffered NaCl solution, and finally organic material in the cell pellet was oxidized with UNO. Pt-

 avoiding uptake of cis-DDP. Cinnamaldehyde, however, induced an opposite, synergistic effect when combined with cis-DDP.

Cinnamaldehyde (4), benzaldehyde (6), and various aliphatic aldehydes (22–24) all inhibit protein synthesis in a variety of in vitro systems. However, since cinnamaldehyde and benzaldehyde modify the effect of cis-DDP in opposite directions, it is not probable that their inhibition of protein synthesis per se is responsible for the modification of the effect of cis-DDP by cinnamaldehyde as reported in this study. For example, benzaldehyde, previously shown to be a protein synthesis inhibitor (7), induced a protective effect in combination with cis-DDP (8). However, this protective effect was independent of an inhibition of protein synthesis. In addition, in (8) we demonstrated that the protein synthesis inhibitor cycloheximide did not have any effect on cell inactivation by cis-DDP.

The present data clearly show that the aldehyde group present in cinnamaldehyde is necessary for the observed potentiation of cis-DDP (Table 1). Furthermore, the synergistic lethal effect seems to occur only when treatment with cinnamaldehyde and cis-DDP is given simultaneously (Fig. 5). The synergistic effect is dose dependent in both cinnamaldehyde and cis-DDP concentrations (Figs. 2 and 3), and present in even up to 4-h treatment periods (Fig. 4). The potentiation of cis-DDP-induced cell inactivation by cinnamaldehyde occurred at all stages of the cell cycle (Fig. 6). Although there is a variability in the fraction of cells surviving cis-DDP treatment, each figure clearly demonstrates a synergistic effect with respect to cell inactivation between cis-DDP and cinnamaldehyde. Differences in survival data for cis-DDP-treated cells may be due to serum effects, for example variations in protein concentration, since cis-DDP does bind to serum proteins and, although we attempt to hold serum composition uniform, our medium E2a may vary with regard to blood serum components. Furthermore, we cannot rule out the possibility that small changes in incubator temperature can result in variations in the survival of cells following cis-DDP treatment.

Although the mechanism causing the synergistic lethal effect of cis-DDP in combination with cinnamaldehyde is not known, two different alternative possibilities must be considered as follows.

Cinnamaldehyde and cis-DDP Could React to Form a Third Compound of Greater Toxicity. Intracellular cis-DDP forms an aquated platinum species which reacts with nucleophilic sites (17). The carbon-carbon double bond present in the propenal moiety of cinnamaldehyde may act as such a site. Preliminary experiments attempting to detect a reaction between cinnamaldehyde and cis-DDP have not shown this to occur during the treatment times used (data not shown).

Uptake of cis-DDP into Cells Could Be Increased by the Simultaneous Presence of Cinnamaldehyde. While benzaldehyde and pyridoxal reduced cis-DDP-induced cell inactivation by apparently limiting cellular uptake of the drug (9, 10), cinnamaldehyde could bind at entirely different sites on the cell membrane and render it more permeable to cis-DDP. Thus the simultaneous presence of cinnamaldehyde could increase cellular uptake of cis-DDP and lead to an enhanced inactivating effect. From Fig. 7, though, no increase in cell-associated platinum was found in cells treated simultaneously with cis-DDP and cinnamaldehyde. Thus, it does not appear that cinnamaldehyde affects cells such that they take up a greater amount of cis-DDP.

Apparently another mechanism is responsible for the synergistic effect of cinnamaldehyde on cis-DDP-induced cell inactivation. A suppression of DNA repair by cinnamaldehyde could seem plausible; however, the results presented in Fig. 5

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demonstrate that synergism occurs only when cis-DDP and cinnamaldehyde are in simultaneous combination. We intend to further investigate the biological and biochemical effects of cinnamaldehyde and drug synergism with cis-DDP.

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