Preferential Inhibition of the Activity of a Stimulatory Protein of Eukaryotic Transcription by Platinum(II) Complexes

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ABSTRACT

The effect of platinum(II) complexes on RNA polymerase II was studied. (o-Glucuronato)(1R,2R-cyclohexanediamine)platinum(II) nitrate (I-GHP) preferentially inhibited RNA synthesis in the presence of S-II, an essential component of eukaryotic transcription. When DNA was pretreated with I-GHP, its template activity decreased significantly, especially when assayed in the presence of S-II. The target of platinum(II) complexes is probably DNA. When DNA is modified, regulatory proteins of transcription, such as S-II, seem to lose their function preferentially on such a template, resulting in the inhibition of RNA synthesis.

INTRODUCTION

Platinum(II) complexes have been shown to have antitumor activity (1, 16). Since these complexes bind tightly to DNA (9, 18), it has been suggested that their primary target is DNA (25). There are several reports showing that these compounds inhibit DNA synthesis in certain tumor cells (3, 15, 23), but little is known about their effects on eukaryotic RNA polymerases. If these compounds bind to DNA, the activity of RNA polymerase should be inhibited, and in fact Srivatava et al. (21) reported that platinum(II) complexes inhibited the activity of Escherichia coli RNA polymerase.

In eukaryotic cells, transcription is a very complicated process. In particular, various protein factors besides RNA polymerase II have been shown to participate in the synthesis of mRNA (8). Recently, we purified a protein named S-II from Ehrlich ascites tumor cells (19). This protein is an essential component of eukaryotic transcription and specifically stimulates the activity of RNA polymerase II in vitro (11, 20, 24, 25).

This paper describes the effect of a cyclohexanediamine isomer of platinum(II) complexes on RNA synthesis in an eukaryotic transcription system, with special attention to its effect on stimulatory protein S-II. This compound has been shown to have antitumor activity and is now being tested clinically (4, 5). We found that this compound preferentially inhibited RNA synthesis in the presence of S-II, suggesting that modification of template by platinum(II) complexes greatly reduces the activity of essential factors rather than that of RNA polymerase II itself in eukaryotic transcription.

MATERIALS AND METHODS

Chemicals. Mono- and bis(o-glucuronato)platinum(II) complexes were prepared as described previously (5) and dissolved in distilled water.

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2 To whom requests for reprints should be addressed.

3 The abbreviation used is: I-GHP, (o-glucuronato)(1R,2R-cyclohexanedi-amine)platinum(II).

.size Analysis of RNA Synthesized in Vitro. RNA synthesis was performed using [α-32P]UTP (10 μCi, 410 Ci/mmol) in the standard assay mixture. Total RNA was extracted with phenol, treated with chloroform, and precipitated twice with 95% ethanol. The resulting precipitate was mixed with 50% (v/v) formamide and 6% (v/v) formaldehyde and incubated at 60° for 7 min. The mixture was subjected to gel electrophoresis on a 1.2% (w/v) agarose containing 6% (v/v) formaldehyde and then to autoradiography (2). Total RNA extracted from Ehrlich ascites tumor cells containing 28S, 18S, and 4S RNA was used as a size marker.

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min at 37°C. Then the mixture was loaded onto a 3.5-ml linear gradient of 12.5 to 25% (v/v) glycerol over a discontinuous gradient of 1 ml of 60% (v/v) glycerol and 0.5 ml of 80% (v/v) glycerol in buffer containing 50 mM Tris-HCl, pH 7.9 (25); 0.1 mM EDTA; 0.04% (v/v) Triton X-100; 0.3 mM dithiothreitol; and 10 mM (NH₄)₂SO₄ and centrifuged at 290,000 x g for 3 hr. Fractions of 8 drops were collected from the bottom of the tube, and those containing DNA were pooled and assayed for template activity. The DNA obtained under these conditions was not contaminated with free I-GHP.

RESULTS

Inhibition of RNA Synthesis by Platinum(II) Complexes.

HeLa cells were incubated with various concentrations of I-GHP for 3 hr and then labeled with [³H]uridine for 30 min. The radioactivity incorporated by a fixed amount of cells was plotted against the concentration of I-GHP. As shown in Chart 1, incorporation of [³H]uridine into the acid-insoluble fraction was almost completely inhibited by the presence of I-GHP (0.3 mg/ml). Since I-GHP inhibited RNA synthesis in vivo, the effects of 5 platinum(II) complexes on RNA polymerase II were tested in vitro to see if they directly inhibited the activity of the enzyme. As summarized in Table 1, all these complexes inhibited RNA polymerase II, but their effective concentrations were significantly higher than that of a specific inhibitor of RNA polymerase II. For instance, α-amanitin, which is known to inhibit RNA polymerase II specifically, is usually effective at a concentration of 1 μg/ml (6). There was no significant difference in the effects of the 5 complexes on RNA polymerase II. Therefore, in subsequent experiments only I-GHP was used.

![Chart 1. Effect of I-GHP on mRNA synthesis of HeLa cells. HeLa S3 cells were incubated with various concentrations of I-GHP for 3 hr and then labeled with [³H]uridine for 30 min. The radioactivity incorporated by a fixed amount of cells was plotted against the concentration of I-GHP. As shown in Chart 1, incorporation of [³H]uridine into the acid-insoluble fraction was almost completely inhibited by the presence of I-GHP (0.3 mg/ml). Since I-GHP inhibited RNA synthesis in vivo, the effects of 5 platinum(II) complexes on RNA polymerase II were tested in vitro to see if they directly inhibited the activity of the enzyme. As summarized in Table 1, all these complexes inhibited RNA polymerase II, but their effective concentrations were significantly higher than that of a specific inhibitor of RNA polymerase II. For instance, α-amanitin, which is known to inhibit RNA polymerase II specifically, is usually effective at a concentration of 1 μg/ml (6). There was no significant difference in the effects of the 5 complexes on RNA polymerase II. Therefore, in subsequent experiments only I-GHP was used.](image)

![Table 1](image)

<table>
<thead>
<tr>
<th>Platinum (II) complex</th>
<th>Dose (mg/ml)</th>
<th>UMP incorporated (pmol)²</th>
<th>Ratio²</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>15.4 (100)²</td>
<td></td>
</tr>
<tr>
<td>cis-GHP</td>
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<td>8.5 (55)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.0 (19)</td>
<td></td>
</tr>
<tr>
<td>I-GHP</td>
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<td>12.0 (78)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.9 (25)</td>
<td></td>
</tr>
<tr>
<td>cis-G₂HP</td>
<td>0.1</td>
<td>10.2 (66)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.2 (21)</td>
<td></td>
</tr>
<tr>
<td>d-G₂HP</td>
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<td>9.1 (59)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.9 (12)</td>
<td></td>
</tr>
<tr>
<td>I-G₂HP</td>
<td>0.1</td>
<td>10.8 (70)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5.7 (39)</td>
<td></td>
</tr>
</tbody>
</table>

¹Means for duplicate determinations.
²Numbers in parentheses, percentage.

Preferential Inhibition of the Activity of the Stimulatory Protein of RNA Polymerase II by I-GHP. We have purified a protein factor named S-II, which is essential for accurate transcription, from Ehrlich ascites tumor cells. This factor was shown to stimulate the activity of RNA polymerase II in vitro. RNA polymerase II synthesized larger RNA in the presence of S-II than in its absence (14). The effect of I-GHP at various concentrations on the activity of S-II was tested. As evident from Chart 2A, RNA synthesis in the presence of S-II was affected significantly by I-GHP. The change in the stimulation ratio of S-II is plotted against the concentration of I-GHP in Chart 2B. I-GHP had no significant effect at concentrations of up to 10 μg/ml, but at higher concentrations the stimulation ratio decreased rapidly with increase in the concentration of I-GHP. These results indicate that S-II is more susceptible to I-GHP than is RNA polymerase II itself.

Many other factors besides S-II are essential for eukaryotic transcription, although these factors have not yet been purified and characterized (8, 17). Manley et al. (7) reported transcription of truncated DNA from a correct initiation site in a HeLa cell lysate. Since RNA synthesis was initiated from the correct initiation site in this system, most of the essential factors necessary for transcription should be present in this lysate. Thus, the effect of I-GHP on accurate transcription was tested in this HeLa cell lysate using a restriction fragment of adenovirus 2 DNA (Smal-F) containing a major late promoter as a template.

As shown in Fig. 1, Lane 1, 536-nucleotide RNA (which is a runoff product from the correct initiation site) was synthesized in this lysate. The synthesis of this RNA was greatly inhibited by I-GHP (100 μg/ml), as shown in Lane 3. Nearly 80% of the RNA polymerase II was still active in the presence of this concentration of I-GHP, as shown in Table 1, indicating that this compound...
preferentially repressed the activity of regulatory proteins such as S-II, rather than that of RNA polymerase II.

**Change in Template Activity of DNA Treated with I-GHP.**

Preferential inhibition of regulatory proteins of RNA polymerase II is probably due to modification of template DNA by platinum(II) complexes. Ehrlich ascites tumor DNA was preincubated with I-GHP (1 mg/ml), and then DNA was separated from free I-GHP by glycerol density gradient centrifugation. The template activities of treated and untreated DNA were compared. As shown in Chart 3A, template activity of treated DNA was about one-half that of untreated DNA, and the difference was greater when template activity was tested in the presence of S-II. As shown in Chart 3B, stimulation of RNA synthesis by S-II was greatly suppressed when treated DNA was used as template. With normal DNA, the stimulation ratio of RNA synthesis by S-II was 3.5- to 5.5-fold depending upon the amount of template DNA, whereas with treated DNA it was less than 2.5-fold. Probably, I-GHP binds directly to DNA or DNA is partially broken down during incubation with I-GHP, resulting in loss of template activity. When DNA was treated with I-GHP (10 mg/ml), its template activity was completely lost (data not shown).

Then, when template is modified with I-GHP, why is RNA synthesis in the presence of S-II preferentially affected? Since the RNA synthesized in the presence of S-II is much larger than that synthesized in its absence, modified template may not be good for synthesis of large RNA. To test this, we examined the molecular size of RNA synthesized in vitro. RNA was labeled with [α-32P]UTP under various conditions and subjected to formaldehyde:agarose gel electrophoresis. As shown in Fig. 2, Lane 3, RNA synthesized in the presence of I-GHP (1 mg/ml) was clearly smaller than control RNA synthesized in its absence. Comparison of Lanes 1 and 4 shows that the molecular size of RNA was larger in the presence of S-II. However, when I-GHP (1 mg/ml) was present, the RNA was significantly smaller, even in the presence of S-II, as shown in Lane 6. Thus, S-II probably
with proteins in the transcriptional machinery. Furthermore, we found that the template activity of DNA pretreated with I-GHP was greatly decreased and that the availability of this template in the presence of S-II was significantly lower than that in its absence. However, it is unknown why S-II does not function properly on modified DNA. Probably, premature termination occurs when S-II is absent and RNA polymerase II fails off from template DNA in the middle of transcription. When S-II is present, however, it stabilizes the elongation complex on template DNA, resulting in the synthesis of a larger RNA molecule, and the elongation complex may not be able to slide smoothly on template DNA when the template is modified by I-GHP. We think that this is the main reason for the preferential inhibition of S-II-dependent RNA synthesis by I-GHP. Thus, the structure of the template seems to be very important for accurate transcription in vivo, and when the structure is modified by I-GHP, the template may not function in the transcriptional machinery.

This situation is the same in both tumor cells and normal cells, and it does not explain the selective toxicity of platinum(II) complexes on tumor cells. It should be pointed out, however, that S-II is a tumor cell protein. S-II may be a modified form of a corresponding protein in normal cells that is less sensitive to I-GHP. In fact, previously, we showed that the amount of S-II per cell, determined by the complement-fixation test, was almost constant in various mouse organs, including Ehrlich ascites tumor cells (22), but that the activity of S-II, monitored as stimulation of RNA polymerase II, was much higher in Ehrlich ascites tumor cells than in tissues of the body (12). We concluded from this work that the stimulatory protein of RNA polymerase II is one of the targets of antitumor activity of platinum(II) complexes. Further studies on this phenomenon may provide a clue to the selective toxicity of these complexes.

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Fig. 2. Size analysis of RNA synthesized in vitro. 35P-labeled RNA synthesized on total mouse DNA in the absence (Lanes 1, 2, and 3) or presence (Lanes 4, 5, and 6) of S-II was extracted and subjected to formaldehyde:agarose gel electrophoresis and then autoradiography. I-GHP was added to the reaction mixture at a concentration of 100 /ig/ml (Lanes 2 and 5) or 1 mg/ml (Lanes 3 and 6). Lanes 1 and 4, RNA synthesized in the absence of I-GHP.

does not function properly in RNA synthesis on template DNA modified by I-GHP.

DISCUSSION

Various types of platinum(II) complexes have been developed as chemotherapeutic agents for cancer, and some of them are now being tested clinically. However, the mechanism of the antitumor activity of these compounds is not understood, although it is known that they bind tightly to DNA.

We investigated the effect of platinum(II) complexes on RNA synthesis. We found that I-GHP inhibited pulse labeling of RNA in HeLa cells, and the same compound was shown to inhibit the activity of RNA polymerase II in vitro. Accurate transcription in a HeLa cell lysate was completely inhibited by I-GHP at a lower concentration than that which affected RNA polymerase II. Moreover, I-GHP inhibited RNA synthesis in the presence of S-II, an essential factor of eukaryotic transcription, to a greater degree than RNA synthesis did by RNA polymerase II alone. These results indicate that I-GHP has more effect in a complete transcription system containing various transcription factors. The preferential inhibitory effect of I-GHP on S-II may be due to modification of template DNA by I-GHP, not to specific interaction between I-GHP and S-II, because the effective concentration of I-GHP was higher than that of a specific inhibitor of RNA polymerase II, and thus it is unlikely that I-GHP interacts specifically

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