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Antitumor activities and interaction with DNA of oxaliplatin-type platinum complexes with linear or branched alkoxyacetates as leaving groups

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Five oxaliplatin-type platinum complexes containing trans-1R, 2R-diaminocyclohexane chelating platinum cores, characteristic of linear or branched alkoxycarboxylates as leaving groups, were biologically evaluated. These compounds showed higher antitumor activity, lower toxicity in vivo than cisplatin or oxaliplatin. And the results revealed that the antitumor activity and interaction with DNA of these compounds were highly related to the nature of leaving groups. Among these complexes, 5a, cis-(trans-1R, 2R-diaminocyclohexane) bis (2-tert-butoxyacetate) platinum(II), showed the highest antitumor activity and the lowest toxicity.

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1. Introduction

In 1969, American scientist Rosenberg firstly found that cisplatin could significantly inhibit proliferation of tumor cells [1–3]. After that, cisplatin became one of the most widely used chemotherapeutics in the treatment of malignant tumors. However, its clinical application was limited by its severe toxicity, narrow range of activity, instinct and acquired drug resistance, and poor aqueous solubility [4–6]. Numerous attempts have been made to develop cisplatin analogs with improved characteristics such as broader spectra of activity, higher clinical efficiency and reduced toxicity [7–14]. Besides, many efforts of developing platinum-based new agents have been made in recent years, such as orally active platinum(IV) complexes, sterically hindered platinum(II) complexes, trans-platinum complexes, multi-nuclear platinum complexes, sulfur-containing platinum complexes, etc. [15]. Although thousands of new complexes were designed and investigated on their activities, only a few of them entered the clinical trials, and less was registered and emerged as the first-line treatment of choice, due to the shortcomings mentioned above [16]. Until now, most clinical platinum drugs are cisplatin analogs. Therefore, the main focus is still aimed at the discovery of cisplatin analogs with improved pharmacological properties. And as part of these analog studies, important structure–activity rules to define the requirements for antitumor activity of platinum compounds have been established [17]. According to these principles, the main strategy to achieve this goal is to modulate the aqueous solubility and liposolubility of potential platinum complexes which are carried out by altering the carrier ligands and/or leaving groups. One is to replace its carrier ligand with enantiomeric amines, and some reports show that this kind of complexes could produce specific diastereoisomeric interactions between the platinum-AA’ moiety and chiral DNA [18–20]. A successful example is clinically approved oxaliplatin which shows no cross-resistance in some cisplatin-resistant cell lines [21]. The lack of cross-resistance is attributed to the trans-1R, 2R-diaminocyclohexane (DACH) carrier ligand [22]. The other way is to substitute the chloride anions of cisplatin by appropriate leaving groups with well-balanced solubility in both water and liposome, since the substitution is greatly helpful to transport drugs into target cells and reduce drug-related toxicities [23–28]. Several platinum drugs have been developed successfully in this way, such as carboplatin and heptaplatin [29].

Based on these previous studies, we have designed and synthesized a series of new cisplatin analogs of the type [PtA2(OCOCR2)2] (where A is a carrier ligand, and R is an alkyl group). The carrier ligand was selected and fixed as trans-1R, 2R-diaminocyclohexane as a result of previous tumor cell toxicity study in vitro [30–32]. And leaving groups were evolved into alkoxyacetate since it has been demonstrated to be an excellent leaving group and be able to improve the aqueous solubility of platinum complexes. Thus, in the present study, we report these platinum complexes (Fig. 1) with attempts to reveal the effect of different leaving groups that are alkoxyacetates with

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2. Experimental section

2.1. Materials and instruments

Human non-small-cell lung cancer cell line A549, human colorectal carcinoma cell line LS-174T and HCT-116, human breast adenocarcinaoma cell line MCF-7, and mouse Lewis lung cancer cell line were purchased from American type Cell Culture (ATCC, Shanghai, China) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and antibiotics. Mice were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. All the complexes were confirmed by IR (Bruker Vector 22 spectrophotometer), 1H NMR (DMSO/TMS, Bruker DRX500 spectroscope) and ESI-MS (Finnigan MAT SSQ 710) spectrometry. All the compounds in the in vivo assay were dissolved at the specific concentration in 5% (w/v) glucose solution (GS), and these solutions were fresh prepared just before the assay started. All reagents and solvents were of analytical reagent grade. Both cisplatin and trans-1R,2R-diaminocyclohexane were purchased from local companies.

2.2. Preparation of complexes and in vitro stability in aqueous solution

Platinum complexes 1a–5a containing alkoxycarboxylate anions (CH3OCH2COO)−(I), CH3CH2OCH2COO)−(II), CH3CH2CH2CH2OCH2COO)−(III), (CH3)2CHOCH2COO)−(IV), (CH3)2COCH2COO)−(V)) were prepared and spectrally characterized as previously reported [30–32]. For stability measurement assay, 1 mg of complex 5a was dissolved in 1 mL water, and incubated at room temperature. And at 0, 20, 40, 60, 90 and 120 min of incubation, 10 μL aliquots were taken and analyzed by RP-HPLC (25% water/75% acetonitrile) on a C18 column (250 mm × 4.6 mm, 5 μm, Diamond) to measure the complex contents in the solution. Then retention percentages were calculated according to the area of peak.

2.3. Antitumor activity study

2.3.1. Tumor implantation

Human non-small-cell lung cancer cell line A549, human colorectal carcinoma cell line LS-174T and HCT-116, human breast adenocarcinaoma cell line MCF-7, and mouse Lewis lung cancer cell line were purchased from American type Cell Culture (ATCC, Shanghai, China) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and antibiotics. Mice were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. All the complexes were confirmed by IR (Bruker Vector 22 spectrophotometer), 1H NMR (DMSO/TMS, Bruker DRX500 spectroscope) and ESI-MS (Finnigan MAT SSQ 710) spectrometry. All the compounds in the in vivo assay were dissolved at the specific concentration in 5% (w/v) glucose solution (GS), and these solutions were fresh prepared just before the assay started. All reagents and solvents were of analytical reagent grade. Both cisplatin and trans-1R,2R-diaminocyclohexane were purchased from local companies.

2.3.2. BALB/c nude mouse treatment

2.4.1. Complexes 1a–3a activity comparing

After the mean A549 and LS-174T tumor volume reached 100–300 mm³, mice were randomly divided into eight groups with six mice per treatment group and ten mice in the negative control group. For A549, group 1 received cisplatin at a dose of 5 mg/kg, group 2 received equal volume injections of 5% GS alone. The other six groups were treated with 1a, 2a or 3a at doses of 5 or 10 mg/kg. For LS-174T, group 1 received oxaliplatin at a dose of 6 mg/kg, group 2 received equal volume injections of 5% GS alone. The other six groups were treated with 1a, 2a or 3a at doses of 5 or 12 mg/kg. All the nude mouse treatment groups were injected i.v. with 0.1 mL of the drug, three times for 14 d (once in 1 d, 5 d and 9 d, respectively).

2.4.2. Complexes 4a and 5a activity comparing

After the mean A549 tumor volume reached 100–300 mm³, mice were randomly divided into eight groups with five mice per treatment group and ten mice in the negative control group. Group 1 received cisplatin at a dose of 5 mg/kg in 5% GS, group 2 received equal volume injections of 5% GS alone. The other six groups were treated with 4a at doses of 7.5, 15 or 30 mg/kg and 5a at doses of 5, 10 or 20 mg/kg, respectively. All the nude mouse treatment groups were injected i.v. with 0.1 mL of the drug, three times for 14 d (once in 1 d, 5 d and 9 d, respectively).

2.4.3. Measurement of tumor growth

Tumors were measured individually with a vernier caliper. Volumes were determined using the formula: tumor volume = length × width × 0.52. Therapeutic effects on tumor growth were expressed as mean tumor volumes versus time, calculated as (1 − T/C) × 100%, where T = treated tumor volume and C = control tumor volume. For example, if treated tumors were 40% of the volume of control tumors on a given day, tumor suppression in the treated group was 60%. At the same time, the weight of each mouse was also measured every other day for 14 d.

2.5. Acute toxicity study

In this work, acute toxicity tests were used to evaluate the safety of these complexes. We first assessed the range of doses of 1a–5a that caused 0% and 100% death rates for mice using a pretest. ICR mice were randomly divided into different groups (5 female mice and 5 male mice for each group) to study the acute toxicity of complexes. The doses of 1a–5a, cisplatin and oxaliplatin were 2.36, 2.62, 2.92, 3.24, and 3.60 mg/kg body wt.; 3.23, 3.43, 3.61, 3.80, and 4.80 mg/kg body wt.; 5.25, 5.85, 7.2, 8.0, and 10.0 mg/kg body wt.; 25.6, 32, 36, 40, 45, 50, 62, and 78 mg/kg body wt.; 3.34, 4.0, 5.0, 5.44, and 6.4 mg/kg body wt.; and 5.0, 6.4, 8.0, 10.0, and 12.5 mg/kg body wt., respectively. All drugs were administered i.v. once daily for 5 d. After administration, all external morphological, behavioral changes, numbers of dead and time to death, as well as some other toxic effects were recorded continuously in 2 weeks. According to the mortality of mice observed within 2 weeks, the LD50 values for each drug were calculated using SPSS software with the Bliss method.
2.6. Induction of cell apoptosis

Human colorectal carcinoma cell line (HCT-116) and human breast adenocarcinoma cell line (MCF-7) were grown in culture. Cell apoptosis assays were performed as follows: briefly, cells were washed with PBS and digested by trypsin solution. A cell suspension was made with culture medium, and the concentration was adjusted to $1 \times 10^5$ cells/mL. Cells were plated into 6-well culture plates (2 mL/well) and incubated at 37 °C in 5% CO$_2$ overnight. A series of indicated doses of test reagents were added into each well and incubated with cells for 24 h at 37 °C in 5% CO$_2$. 5% GS was used as a negative control; cisplatin and oxaliplatin were used as positive controls for MCF-7 and HCT-116, respectively. The apoptosis of cells was measured by Flow cytometry using annexin V-FITC/PI apoptosis kit (Biouniquer, Nanjing, China) according to the manufacturer’s instructions as below. Cells were harvested and washed in cold PBS, then stained with annexin V-FITC (100 ng/mL) and propidium iodide (2 μg/mL) in annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl$_2$, pH 7.4). After 15 min incubation at room temperature, the fluorescence of cells was measured using the flow cytometer (FAC Scan, Becton Dickenson, USA). The results were analyzed using Cell Quest Pro software and represented as percentage of normal and apoptotic cells at various stages [33]. FITC and PI fluorescences were measured in FL1 and FL2 channels, respectively.

2.7. Gel electrophoresis experiment

Interaction of the platinum complexes with pET22b plasmid DNA was studied by agarose gel electrophoresis. Cisplatin and oxaliplatin were used as controls. Briefly, Super coiled pET22b plasmid DNA (at concentration 0.5 μg/mL) were incubated with different platinum complexes in Tris buffer (50 mM Tris-acetate, 18 mM NaCl buffer, pH 7.2) in a water bath at 37 °C in the dark for 4 h. Then 10 μL aliquots of drug–DNA mixtures were loaded onto the 1.0% agarose gel stained with GoldenView and electrophoresis was carried under TAE buffer (0.05 M Tris base, 0.05 M glacial acetic acid, 1 mM EDTA, pH=8.0) for 30 min at 100 V. At the end of electrophoresis, the gels were then photographed under UV light (Gel Doc XR Imaging System, Bio-Rad). The quantity of each band was given as its total optical density (OD value), as amount of total gray. The OD values were measured experimentally, and the other values were calculated using Quantity One software [Bio-Rad]. The experiments were repeated three times.

2.8. Statistical methods

Data are expressed as mean±SD. Statistical significance was assessed using the Student t test. For all statistical comparisons, treated groups were compared with PBS-treated controls, and $P<0.05$ or $P<0.01$ was considered statistically significant or extremely significant, respectively.

3. Results

3.1. The preparation of designed platinum(II) complexes

All complexes were designed and synthesized in our previous studies, and the structures of the complexes were confirmed by IR, 1H NMR, and ESI-MS spectra. All spectral peaks of these compounds were compatible to the related molecular structures given in Fig. 1 [30–32]. And the representative of platinum(II) complexes, 5a, showed certain stability in aqueous solution (Fig. S and Table S).

3.2. Antitumor activity of the synthesized platinum(II) complexes in vivo

For in vivo assay, the mice (C57BL/6) implanted with Lewis lung cancer cells were injected s.c. with the complexes to perform the preliminary screening. We assessed 2a and 3a at three doses of 2.5 mg/kg, 5 mg/kg and 10 mg/kg in the treatment and discovered that 5 mg/kg and 10 mg/kg were the most effective on the Lewis carcinoma (data not shown). Subsequently, we did the further study to examine their effects on tumor-bearing nude mice.

Comparing the activity of complexes 1a–3a in the in vivo study, the inhibitory effect of complexes 1a–2a on the tumor growth was dose-dependent, while complex 3a exhibited a different dose–effect manner. These complexes showed different sensitivities towards two human cancers, A549 (Fig. 2A) and LS174T (Fig. 2B). In A549

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Therapeutic effects of Pt complexes 1a–3a on the growth of human non-small-cell lung carcinoma (A549) in vivo.</strong></td>
</tr>
<tr>
<td>Group</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>1a</td>
</tr>
<tr>
<td>2a</td>
</tr>
<tr>
<td>3a</td>
</tr>
<tr>
<td>Cisplatin</td>
</tr>
<tr>
<td>Cisplatin</td>
</tr>
</tbody>
</table>

NS: 5% GS without complexes.

* $P<0.05$.

** $P<0.01$.
3.3. Acute toxicity of the platinum(II) complexes in vivo

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>RTV (± SD)</th>
<th>T/C (%)</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>NS</td>
<td>15.26 ± 5.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>6</td>
<td>8.93 ± 2.95</td>
<td>58.5</td>
<td>41.5</td>
</tr>
<tr>
<td>2a</td>
<td>6</td>
<td>5.06 ± 0.31</td>
<td>31.2</td>
<td>66.8</td>
</tr>
<tr>
<td>3a</td>
<td>6</td>
<td>4.48 ± 1.84</td>
<td>29.4</td>
<td>70.6</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>6</td>
<td>7.75 ± 2.31</td>
<td>50.8</td>
<td>49.2</td>
</tr>
</tbody>
</table>

NS: 5% GS without complexes.
* P< 0.05.
** P< 0.01.

Fig. 3. The inhibition activity of Pt complexes 4a and 5a on the growth of human non-small-cell lung carcinoma in vivo (*P<0.05).

treatment groups, the significant antitumor effects (P<0.05) were only observed at high dose (10 mg/kg) of complexes 1a and 2a, and the inhibition rates were 48% and 53.1%, respectively (Table 1). The sizes of implanted human colon cancer (LS-174T) in the groups treated with synthesized complexes 1a–3a were all significantly smaller than that in the 5% GS control group. 41%, 66.8% and 49.2% inhibitions of tumor volume after 14 days' complexes 1a–3a treatment (P<0.05 or P<0.01) were observed at the dose of 12 mg/kg, respectively (Table 2). In in vivo study of comparing activity of complexes 4a and 5a, the A549 tumor volumes of 5a group were significantly smaller than the control group at both dosages of 15 mg/kg and 30 mg/kg (P<0.05), and the highest inhibition rate was 66.5% (Fig. 3). Additionally, complex 4a could also significantly inhibit tumor growth (66.2%) at the high dosage (P<0.05).

3.4. Platinum(II) complexes induced cell apoptosis

The exposure of MCF-7 (Fig. 4, F–I) and HCT-116 cells (Fig. 4, A–D) to the test compounds assumed various degrees of apoptosis. The dot plot shows three distinct populations: a) the viable cells which have low Annexin V-FITC and low PI signal (Q3 in panel); b) the apoptotic cells which have high Annexin V-FITC and low PI signal (Q4 in panel); c) late stage apoptotic/secondary necrotic cells with compromised membranes exhibiting high Annexin V-FITC and high PI signal (Q2 in panel). In some cases, a fourth population corresponding to damaged viable cells with low Annexin V-FITC and high PI signal may be observed (Q1 in panel). In general, the complex treated cells exhibit a higher percentage of apoptotic cells than that seen in the untreated (Control) cells. Complexes 4a and 5a at 50 μM dose induced apoptosis of MCF-7 cells in 40.7% and 49.6% (Fig. 5), respectively, which behaves like cisplatin (56.6%). While oxaliplatin could not obviously induce cell apoptosis until the dosage was increased to 100 μM which was double as much as the other complexes. Besides, complexes 4a and 5a could also induce marked apoptosis of HCT-116 cells which exhibit strong drug resistance to cisplatin, and the apoptosis rates of 4a and 5a were same as double-dosed oxaliplatin (35%).

3.5. Platinum(II) complex reaction on DNA

Considering that DNA platination is a necessary condition for cytotoxic activity of platinum complexes, the reaction on plasmid DNA of complexes 4a and 5a was generally monitored by agarose gel electrophoresis. In this experiment, plasmid DNA was mainly in supercoiled form, so it showed only one band in gel. When the test compounds were incubated with plasmid DNA at 37 °C, they could coordinate to the DNA molecule which made DNA molecule more fragile to outside circumstance. As a result, the plasmid DNA coordinated to outside circumstance. As a result, the plasmid DNA could also induce marked apoptosis of HCT-116 cells which exhibit strong drug resistance to cisplatin, and the apoptosis rates of 4a and 5a were same as double-dosed oxaliplatin (35%).

4. Discussion

In our previous work, chloride anions were replaced by diverse carboxylate anions to promote the aqueous solubility of the related platinum complexes. And the results indicated that most of them showed good in vitro cytotoxicities against the selected cell lines in addition to appropriate solubility [30,34,35].

Table 3

<table>
<thead>
<tr>
<th>Complex</th>
<th>LD50 and 95% FL (mg/kg)</th>
<th>LD95 and 95% FL (mg/kg)</th>
<th>LD50 and 95% FL (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>2.85</td>
<td>2.64</td>
<td>3.08</td>
</tr>
<tr>
<td>2a</td>
<td>2.63-3.09</td>
<td>2.33-2.99</td>
<td>2.72-3.48</td>
</tr>
<tr>
<td>3a</td>
<td>3.46</td>
<td>3.17</td>
<td>3.77</td>
</tr>
<tr>
<td>4a</td>
<td>3.29-3.63</td>
<td>2.87-3.51</td>
<td>3.46-4.11</td>
</tr>
<tr>
<td>5a</td>
<td>6.05</td>
<td>4.77</td>
<td>7.67</td>
</tr>
<tr>
<td>6a</td>
<td>5.01-7.3</td>
<td>3.29-6.92</td>
<td>5.63-10.46</td>
</tr>
<tr>
<td>7a</td>
<td>32.49</td>
<td>28.28</td>
<td>37.32</td>
</tr>
<tr>
<td>8a</td>
<td>24.95-38.34</td>
<td>10.59-31.66</td>
<td>33.53-81.41</td>
</tr>
<tr>
<td>9a</td>
<td>66.44</td>
<td>52.37</td>
<td>84.31</td>
</tr>
<tr>
<td>10a</td>
<td>60.80-73.61</td>
<td>41.82-57.85</td>
<td>75.53-109.62</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>4.27</td>
<td>3.46</td>
<td>5.26</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>3.74-4.86</td>
<td>2.78-4.29</td>
<td>4.39-6.30</td>
</tr>
</tbody>
</table>
On the basis of these findings, we previously prepared complexes 1a–3a, and antitumor activity of these compounds exhibited high cytotoxicity against a number of tumor cell lines in vitro in our previous study [30]. Thus, these three compounds were used for further investigations. The results of in vivo study showed that these compounds displayed high antitumor activities, and were also effective in the tumor which developed drug resistance to cisplatin. Besides, we also found that these three complexes had better aqueous solubility compared with cisplatin, oxaliplatin and carboplatin. The results suggested that alkoxyacetates as leaving groups could conspicuously promote the aqueous solubility of platinum complexes, which was helpful to transport drugs into target cells to improve the antitumor activity of complexes. At the same time, alkyl groups could balance the liposolubility with aqueous solubility, which was helpful to transport drugs into target cells to improve the antitumor activity of complexes. From the results of in vitro assays, the antitumor activity order was 2a > 1a > 3a, suggesting the liposolubility was not linearly correlated with effect.

In view of the importance of toxic and side effects of complexes in developing new drugs, we examined in vivo acute toxicity of compounds 1a–3a compared with cisplatin and oxaliplatin. Based on the comparison of LD50 values of these compounds, the structure-toxicity relationship revealed that the structure of the leaving groups was very important to side-effect of compounds. From the data, it was found that complexes 1a and 2a not only were the most effective but also had highest toxicity.

To overcome this shortcoming, we alternated the leaving groups by replacing linear alkyl groups with branched ones, named 4a and 5a. And the two complexes were less toxic than cisplatin and oxaliplatin. The results indicated that Pt complexes with branched alkyl groups displayed more well-balanced solubility in water and liposome than linear ones, which is helpful to improve antitumor activity as well as reduce side-effect. Subsequently, we focused on the mechanism of cytotoxicity of complexes 4a and 5a. Results indicated that complexes 4a and 5a showed not only better antitumor activity than oxaliplatin, but also broader antitumor spectra than cisplatin. And the DNA cleavage ability of complexes suggested that different cytotoxicities of complexes might be due to diversity of DNA affinity and cleavage ability. In accordance with structures of those platinum complexes, branched alkoxyacetates as leaving groups might cause relatively higher DNA affinity than linear ones, which may further produce more DNA adducts and may be responsible for the increased DNA cleavage ability and the higher cytotoxicity of the compounds. Although the interaction of these compounds with the pET22b plasmid DNA was observed as approximately effective as cisplatin, these compounds exhibit different antiproliferation activities with cisplatin against the various cell lines used based on the previous study. This suggested that DNA platination may not be the sole mechanism that determines their cytotoxicity and specific mechanism studies are in progress for further investigation. In consideration of high activity and low toxicity of complex 5a relative to cisplatin, it might provide a new model for the development of platinum(II) complexes designed to target and treat a variety of cancers in a specific manner.

5. Conclusions

In this study, most compounds not only exhibit better antitumor activity than cisplatin and oxaliplatin against tested tumors, but also possess desirable physico-chemical properties such as rather good solubility in both water and organic solvents. Complex 5a, cis-[trans-1R, 2R-diaminocyclohexane] bis (2-tert-butoxoacetate) platinum(II), presents pretty high antitumor activity and fairly low toxicity. Therefore, it might be a potential antitumor candidate and worth further investigation. Simultaneously, though complexes 1a and 2a showed some toxic effect, they exhibited higher antitumor activity than cisplatin and oxaliplatin, and also showed no cross-resistance with cisplatin and oxaliplatin, suggesting they have broader antitumor spectra. Thus, these compounds might be applied to different tumors.
Abbreviations

PBS  phosphate-buffered saline
W/V  weight per volume
DACH 1,2-diaminocyclohexane
GS  glucose solution
TMS  tetramethyl silane
ESI-MS  electrospray ionization-mass spectrometry
RP-HPLC  reverse phase high-performance liquid chromatography
FITC  fluorescein isothiocyanate
PI  propidium iodide

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