Cytotoxic Analogs of the Iron(III) Chelator Pyridoxal Isonicotinoyl Hydrazone: Effects of Complexation with Copper(II), Gallium(III), and Iron(III) on Their Antiproliferative Activities

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This study examined if complexation with metals increased the antiproliferative activities of chelators of the pyridoxal isonicotinoyl hydrazone (PIH) class. Addition of iron(III) to some PIH analogs markedly depressed their activities, whereas it had little effect on others. The gallium(III) complex of PIH, but not its copper(II) complex, was more efficient than the apochelator at inhibiting [3H]thymidine incorporation.

Iron (Fe) is a crucial component of a variety of metabolic pathways that are involved in DNA synthesis and the production of energy. Neoplastic cells have a high Fe requirement due to their rapid rate of proliferation (9, 12). Hence, this metal ion is a particularly good target to remove from tumor cells to inhibit replication. A number of clinical trials have shown that the therapeutically used Fe chelator desferrioxamine (DFO) can very effectively inhibit the growth of tumors, such as leukemia and neuroblastoma (NB) (4, 6). Recently, we have identified a number of novel Fe-chelating agents of the pyridoxal isonicotinoyl hydrazone (PIH) class that demonstrate high activity at inhibiting the growth of a range of tumor cells, including NB, leukemia, and melanoma cell lines (11-13). These chelators were far more effective than DFO and showed antiproliferative activities comparable to cis-platin and bleomycin (11). In addition, the ligands displayed pronounced efficacy at preventing 59Fe uptake from transferrin and mobilizing 59Fe from prelabelled cells (11). The PIH class of compounds are tridentate ligands that have been shown to be comparable to DFO in terms of their ability to strongly bind Fe(III) (18). Furthermore, the specificity of these chelators for Fe(III) is also very similar to that of DFO and much greater than that of EDTA (10). In the present study we have investigated the effects of several different metal complexes of the PIH analogs on cellular growth, as metal complexes of a number of other chelators show much greater antiproliferative activities than the ligands alone (7, 8).

The PIH analogs were synthesized by Schiff base condensation between three aromatic aldehydes (pyridoxal, 100 series; salicylaldehyde, 200 series; 2-hydroxy-1-naphthylaldehyde, 300 series) and a number of acid hydrazides by standard procedures (7). In the present investigation, five PIH analogs previously shown (13) to be highly effective at preventing cellular proliferation were examined. These chelators were 206, 305, 308, 311, and 315 (Fig. 1). These ligands were characterized as described previously (5). DFO was obtained from Ciba-GEIGY Pharmaceutical Co. (Summit, N.J.). In some experiments, the Fe, copper (Cu), and gallium (Ga) complexes of the chelators were prepared by adding an equimolar amount of Fe(III) (as FeCl3), Cu(II) (as CuCl2), or Ga(III) (as Ga(NO3)3) to the ligands dissolved in minimum essential medium. The solutions were then mixed thoroughly and incubated for 1 h at 37°C prior to their addition to cell cultures. Complexation was monitored spectrophotometrically between 200 and 800 nm.

The human NB cell line SK-N-MC was obtained from the American Type Culture Collection (Rockville, Md.) and was grown, subcultured, and prepared for experiments as described by Richardson and Milnes (11). Cellular enumeration was determined with an improved Neubauer counting chamber, and viability was assessed by trypan blue staining (11). The synthesis of DNA was measured indirectly by quantitating [methyl-3H]thymidine incorporation in the presence and absence of the agents to be tested, as described before (11). In these studies examining the effects of agents on growth or DNA synthesis, cells were seeded at 5.333 × 10^4 cells/cm² and allowed to grow overnight before addition of the compounds to be tested (11). This seeding density resulted in exponential growth of cells in chelator-free medium throughout the duration of the assay (11).

Our earlier results have shown that the PIH analogs are highly effective at mobilizing 59Fe from cells and preventing 59Fe uptake from transferrin, even at very low ligand concentrations (11-13). Hence, the antiproliferative effects of these agents may be related to their ability to bind Fe. However, when Fe was preincubated with each compound at an equimolar ratio, a variable response was obtained (Fig. 2). The addition of Fe to DFO markedly inhibited its antiproliferative effect on NB cells, while preincubation of analogs 206, 311, and 315 with Fe had little influence on their activities (Fig. 2). In contrast, the addition of Fe to 305 and 308 largely prevented their antiproliferative effects (Fig. 2). The ability of Fe to prevent the activity of DFO is well known (1, 2), and in the present study DFO was used as an effective control. Further, the addition of an equimolar amount of Fe to a range of DFO concentrations (1 to 500 μM) prevented its antiproliferative effect in each case (data not shown). The difference in the ability of Fe to prevent the antiproliferative activities of the PIH analogs was surprising, as this group of compounds all have the same Fe-ligating site (i.e., carbonyl oxygen, aldimine nitrogen, and hydroxyl oxygen [Fig. 1]). The finding that com-
plexation with Fe did not markedly inhibit the effects of ligands such as 206 may indicate that these Fe complexes are not chemically inert and could generate cytotoxic oxygen radicals, as seen with other chelators (16). Alternatively, the Fe complexes of these analogs may be cytotoxic by other mechanisms apart from their ability to chelate Fe. Regarding these possibilities, it is known that the cytotoxic agent bleomycin is an Fe chelator that binds Fe and then undergoes a redox cycling process to generate reactive oxygen species that damage DNA (3). Obviously, further studies are required to determine if a similar process is involved for some of the Fe complexes of the PIH analogs (e.g., 206). Previous work on the Fe complex of 1-formylisoquinoline thiosemicarbazone showed that this chelate could directly inhibit ribonucleotide reductase, the enzyme necessary for the conversion of ribonucleotides into deoxyribonucleotides for DNA synthesis (17). Since the thiosemicarbazone group of chelators bear structural resemblance to the PIH analogs, it is not unreasonable to suggest that further experiments should also examine the effect of the PIH analog Fe complexes on ribonucleotide reductase activity.

Considering the results above, it was deemed worthwhile to examine if complexes with Ga(III) could inhibit proliferation more effectively than complexes with Fe. The Ga(III) ion has antiproliferative activity by itself, as it has many chemical similarities with Fe(III), resulting in a marked disturbance of intracellular Fe metabolism (15). In the present investigation, the addition of an equimolar amount of Ga(III) to PIH potentiated its ability to inhibit [3H]thymidine uptake by SK-N-MC NB cells over a 24- and 48-h incubation (e.g., after a

![FIG. 1. Structures of the five PIH analogs examined in the present study: salicylaldehyde p-t-butybenzoyl hydrazone (206), 2-hydroxy-1-naphthylaldehyde p-aminobenzoyl hydrazone (305), 2-hydroxy-1-naphthylaldehyde m-chlorobenzoyl hydrazone (308), 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311), and 2-hydroxy-1-naphthylaldehyde 2-thiophenecarboxyl hydrazone (315). The structures of these ligands are compared to DFO and to the parent compound, PIH.](http://aac.asm.org/figure/1-structures-of-the-five-pih-analogs-examined-in-the-present-study-salicylaldehyde-p-t-butybenzoyl-hydrazone-206-2-hydroxy-1-naphthylaldehyde-p-aminobenzoyl-hydrazone-305-2-hydroxy-1-naphthylaldehyde-m-chlorobenzoyl-hydrazone-308-2-hydroxy-1-naphthylaldehyde-isonicotinoyl-hydrazone-311-and-2-hydroxy-1-naphthylaldehyde-2-thiophenecarboxyl-hydrazone-315-the-structures-of-these-ligands-are-compared-to-dfo-and-to-the-parent-compound-pih.)

![FIG. 2. The effects on antiproliferative activity of adding an equimolar amount of iron (as ferric chloride) to DFO and the PIH analogs 206, 305, 308, 311, and 315. Exponentially growing SK-N-MC NB cells were incubated with the chelators and their iron complexes for 48 h at 37°C. The results are means of duplicate determinations from a typical experiment of three experiments performed. Variation between replicates was less than 5%. Note that DFO was examined at a concentration of 55 μM, whereas the PIH analogs were tested at 2.2 μM because of their higher antiproliferative activities. CON, control.](http://aac.asm.org/figure/2-the-effects-on-antiproliferative-activity-of-adding-an-equimolar-amount-of-iron-as-ferric-chloride-to-dfo-and-the-pih-analogs-206-305-308-311-and-315-expontially-growing-sk-n-mc-nb-cells-were-incubated-with-the-chelators-and-their-iron-complexes-for-48-h-at-37-c-the-results-are-means-of-duplicate-determinations-from-a-typical-experiment-of-three-experiments-performed-variation-between-replicates-was-less-than-5%-note-that-dfo-was-examined-at-a-concentration-of-55-μm-whereas-the-pih-analogs-were-tested-at-2.2-μm-because-of-their-higher-antiproliferative-activities-con-control.)
The effects of PIH and salicylaldehyde p-t-butylbenzoyl hydrazone (206) in the presence or absence of gallium(III) nitrate on $[^3H]thymidine incorporation by SK-N-MC NB cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µM) 24 h</th>
<th>IC$_{50}$ (µM) 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIH</td>
<td>38</td>
<td>27</td>
</tr>
<tr>
<td>PIH + Ga(III)</td>
<td>22</td>
<td>10</td>
</tr>
<tr>
<td>206</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>206 + Ga(III)</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>Ga(III)</td>
<td>50</td>
<td>23</td>
</tr>
</tbody>
</table>

* Results are means of four determinations in a typical experiment. Cells were incubated for 24 or 48 h in the presence of the listed agents.

48-h incubation the 90% inhibitory concentration (IC$_{90}$) decreased from >50 to 23 µM [Table 1]). However, when Ga(III) was added to PIH analog 206, no potentiation was seen; in fact, it slightly reduced the efficacy of 206 at preventing $[^3H]thymidine uptake in SK-N-MC NB cells after a 48-h incubation (PIH IC$_{50}$ = 28 µM; PIH plus Cu(II) IC$_{50}$ = 29 µM). The addition of Cu(II) to PIH had little effect on its ability to prevent $[^3H]thymidine uptake in SK-N-MC NB cells after a 48-h incubation (PIH IC$_{50}$ = 28 µM; PIH plus Cu(II) IC$_{50}$ = 29 µM). The addition of Cu(II) to analog 206 only very slightly potentiated its inhibitory effect, resulting in a decrease in the IC$_{50}$ from 0.6 to 0.3 µM after a 48-h incubation. For all studies examining the effects of the metal ions on the activity of the chelators, it should be noted that repeat experiments demonstrated that the results for each compound were not variable. Rather, distinct variability in response to the metal was seen between the ligands.

Collectively, the above data obtained with Fe(III), Ga(III), and Cu(II) suggest that the biological activities of the metal complexes appear to differ between the individual PIH analogs, despite their having the same metal-binding site. This difference in activity could result from subtle changes caused by the substitutions distal to the common metal ion-binding site in these analogs (Fig. 1). For example, changes in electron distribution caused by electron-withdrawing or -donating substituents could have a marked effect on the types of metal complexes that are formed and thus explain the difference in biological activity. This suggestion is substantiated by previous studies with the PIH analogs which showed that substitutions distal to the metal-binding site could influence their acid ionization constants (14).

Further studies examining the mechanism of action of these complexes together with work investigating the antiproliferative effects of a range of PIH analogs complexed with other metal ions, such as palladium or zinc, may be worthwhile in terms of identifying useful cytotoxic agents.

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REFERENCES