Biochemical Pharmacology, Vol. 52, pp. 65–71, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00139-6

The 2,2′-Bipyridyl-6-Carbothioamide Copper (II) Complex Differs from the Iron (II) Complex in its Biochemical Effects in Tumor Cells, Suggesting Possible Differences in the Mechanism Leading to Cytotoxicity

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ABSTRACT. 2,2′-bipyridyl-6-carbothioamide (BPYTA) is an antitumor agent with chelating properties. It has previously been shown that the R2 subunit of ribonucleotide reductase (RR) is its major cellular target, but RR inhibition is observed only in the presence of ferrous iron (BPYTA-Fe, molar ratio 2:1). Because the copper (II) complex of BPYTA (BPYTA-Cu, molar ratio 1:1)) has *in vitro* antitumor activity comparable to that of BPYTA-Fe, we studied the mechanism of action of this new metal complex. Spectorphotometric and HPLC studies demonstrated that, at pH 7.5, BPYTA-Cu is stable at molar ratio 2:1 and copper is in its favored oxidized form [BPYTA-Cu(II)]. Electron paramagnetic resonance (EPR) studies with mouse recombinant R2 demonstrated that BPYTA-Cu destroys the R2 tyrosyl radical at the same concentration at which BPYTA-Fe does (78% vs 73% destruction at 200 μM, with 5 min of contact), but R2 inhibition is not time-dependent. Studies of the metabolism of [^{14}C] cytidine suggest that the cytotoxic activity of BPYTA-Cu can be explained in terms of RR inhibition. However, the significant inhibition of RNA synthesis and the lack of cross-resistance to BPYTA-Cu of cell lines resistant to other RR inhibitors suggest that BPYTA-Cu may have more than one cellular target. Moreover, cell proliferation studies suggest that, unlike BPYTA-Fe, BPYTA-Cu displays its activity immediately after contact with the target cells. Our study demonstrates that significant differences in the biochemical effects of BPYTA and, perhaps, also its mechanism of action are due solely to the bonded transition metalloelement. This might also be the case with other chelators that demonstrate cytotoxic activity following metalloelement chelation. BIOCHEM PHARMACOL 52;1:65–71, 1996.

KEY WORDS. 2,2′-bipyridyl derivatives; chelators; ribonucleotide reductase inhibitors; ribonucleotide reductase R2 subunit; copper; *in vitro* antitumor activity

BPYTA† has antitumor activity [1,2]; it has structural and functional similarities with α-HCATs [1–3]. Its main cellular target is the R2 subunit of RR, the enzyme responsible for converting ribonucleoside diphosphates into the deoxyribonucleotide precursors of DNA [3–5]. This inhibition is reversible and takes place only in the presence of Fe(II), with which BPYTA forms a stable complex [1].

BPYTA can form complexes with other metalloelements, such as Cu(II) (BPYTA-Cu) and Zn(II) [6, 7]. Because copper complexes of α-HCATs and some other metalloelement-chelating compounds have been found to have higher antitumor activity than the free ligand and the iron complex [8, 9], it was felt that BPYTA-Cu warranted further investigation.

Our study suggests that the mechanism of action of BPYTA-Cu is different from that of BPYTA-Fe. In fact, BPYTA-Cu seems to have more than one cellular target, by analogy with some copper complexes of α-HCATs [10–12].

Some of the results presented below have been reported elsewhere [13].

MATERIALS AND METHODS

Drugs and Chemicals

BPYTA and its iron (II) and copper(II) complexes were synthesized as previously reported [2, 6, 7]. The commercial preparation of hydroxyurea (HU, Oncocarbide, Simes) was used. RPMI-1640, Dulbecco's modified Eagle's medium, sera, and additive solutions were purchased from Biochrom-Seromed (Berlin, Germany). ^{125}IUdR, 35.4 Ci/mmole, was

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† *Abbreviations:* BPYTA, 2,2′-bipyridyl-6-carbothioamide; α-HCATs, α-(N)-heterocyclic carboxyaldehyde thiosemicarbazones; RR, ribonucleotide reductase; BPYTA-Cu, complex BPYTA-Cu; HU, hydroxyurea; ^{125}IUdR, ^{125}I-5-iodo-2′-deoxyuridine; EPR, electron paramagnetic resonance; BPYTA-Fe, complex BPYTA-Fe.

Received 4 July 1995; accepted 12 February 1996.

purchased from Amersham Italia (Milan, Italy). Cytidine-2-^{14}C, 55.8 mCi/mmole, and other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Drug Solubilization

HU, BPYTA-Fe, and BPYTA-Cu were dissolved in bidistilled water; BPYTA was solubilized to a concentration of 18 mM in DMSO. The highest DMSO concentration used (0.2%) had no cytotoxic effect in our test systems.

Cell Culture

The following cell lines were used: P388, murine leukemia; P388-R made resistant to BPYTA by treating P388 with the drug [14, 15]; TA3, murine mammary adenocarcinoma; TA3H2, made resistant to HU by treating TA3 with the drug [16]. The P388 cell line was purchased from the American Type Culture Collection, and TA3 and TA3H2 were kindly provided by Prof. Lars Thelander (Department of Medical Biochemistry and Biophysics, University of Umeå, Sweden).

Cells were grown exponentially in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The P388 and P388-R cell lines were maintained in RPMI-1640 medium containing antibiotics (penicillin G, 100 Units/mL; streptomycin, 100 μg/mL; gentamicin, 50 μg/mL) and supplemented with 3 mM glutamine, 10 mM HEPES buffer (pH 7.3), 0.01 mM 2-mercaptoethanol and 15% heat-inactivated newborn calf serum. Wild-type TA3 and TA3H2 cell lines were maintained in Dulbecco's modified Eagle's medium containing antibiotics (penicillin G, 100 Units/mL; streptomycin, 100 μg/mL; gentamicin, 50 μg/mL) supplemented with 10% heat-inactivated horse serum and 3 mM glutamine. The TA3H2 cells were continuously exposed to 2 mM HU during routine passages, but the drug was omitted at least 4 days prior to experiments.

Antiproliferative Assay

The assay used to evaluate cytotoxic activity on P388 and P388-R cell lines was developed for the predictive evaluation of tumor chemosensitivity [17] and was previously used in *in vitro* studies of new compounds with potential antitumor activity [18]. Briefly, various concentrations of each drug (in quadruplicate) were placed with tumor cell suspensions in tissue culture plates (37°C; 0.2 mL; 10^4 cells/well) for continuous exposure (48-hr incubation). Alternatively (pulse contact), cell suspensions were incubated with various concentrations of each compound in 4 mL tubes (37°C, 1 mL P388, 5×10^4 cells/mL). After 1 hr of contact, cells were washed twice by centrifuging and placed (in quadruplicate) in tissue culture plates (37°C; 0.2 mL; P388, 10^4 cells/well).

After 48-hr incubation, DNA synthesis was evaluated by adding ^{125}IUdR (0.1 μCi/well) together with 2'-deoxy-5-fluorouridine (0.01 μg/well) to the cultured cells for an additional 18 hr. During this time, the cells continued to grow exponentially. Harvesting was performed with a multiple suction filtration apparatus (Mash II) on a fiberglass filter (Whittaker Co., Walkersville, MD, U.S.A.). Paper disks containing aspirated cells were counted in a gamma-scintillation counter. For each concentration of compound tested, cell growth inhibition was expressed as the percentage of inhibited radioisotope incorporation in treated cultures compared with nontreated controls.

A different assay was used to evaluate the cytotoxic activity of BPYTA and its complexes against wild-type TA3 and TA3H2 cell lines [19]. Logarithmically growing cells were treated with trypsin-EDTA and the cell suspension was plated in 6-cm dishes (10^5 cells/dish). After these cells had attached (6 hr later), various concentrations of each drug were added in duplicate (5-mL medium/dish, in total). After 66 hr, to determine effects on growth, dishes were washed twice and the cells treated with 2 mL of 0.1 M sodium hydroxide for 15 min at 50°C. Absorbance at 260 nm was considered a quantitative measure of cell density [20]. Results are expressed as the percentage ratio of $A_{260\ nm}$ in the treated cultures vs nontreated controls.

EPR Spectroscopy

Tyrosyl free-radical concentration in mouse recombinant R2 preparations [21] was determined by EPR spectroscopy at 77 K, with a microwave power of 20 mW, using a Bruker ESP 300E model and an E-238 cavity. Experiments were performed as follows. Frozen R2 apoprotein (a generous gift from Prof. Thelander) was concentrated, desalted, and dissolved in a solution of 50 mM Tris(hydroxymethyl)aminomethane HCl (pH 7.5) and 100 mM KCl. It was then reactivated with iron, as previously reported [21, 22], passed over a Sephadex G-25 column, balanced with the same R2 buffer (column:sample volume, 10:1) to remove the non-incorporated iron and, then, stored at −70°C at a concentration of 15 μM. Immediately before the experiment, it was rapidly thawed, aliquoted in EPR tubes (200 μL), and maintained in ice. After the addition of a few microliters of buffer or drug solution, these tubes were incubated in a water bath at 37°C. After the required interval, they were refrozen and stored in liquid nitrogen until radical concentrations were again measured. Every thawing procedure resulted in a signal decrease of 10% in the control EPR spectra, so no more than 2 thawings were performed for each sample. The decrease in tyrosyl-radical concentration caused by these compounds was quantified by comparing spectra peak heights of the treated sample and the non-treated control and expressed as a percentage. The absolute radical concentration (5–7 μM) was quantified by comparing spectra peak heights, using a standard mouse R2 protein sample of known tyrosyl free-radical content [21].

Spectrophotometric Studies

Absorption spectra were obtained with a Beckman spectrophotometer. All experiments were performed at a con-

trolled temperature of 25°C. Drugs were dissolved in 100 mM acetate buffer (pH 5) or in a 10 mM Tris(hydroxymethyl)aminomethane KCl buffer (pH 7.5).

HPLC Studies

An isocratic HPLC method for separating BPYTA complexes on a reversed-phase column was used. The column was a 150 × 4.6 mm ID (5 μM packing) Spherisorb (Phase Sep). Mobile phase: 0.2 M triethanolamine pH 3 with $HClO_4$; 15% methanol and 15% acetonitrile. The sample (10–20 μg/mL) was injected using a 50 μL loop at ambient temperature with a flow rate of 1 mL/min and detection at 254 nm. Under these conditions, the retention time (RT) for BPYTA was 6.07 min, for BPYTA-Cu 3.73 min, and for BPYTA-Fe 2.72 min.

Inhibition of Ribonucleotide Reductase Activity in Intact Cells

The following method, developed in our laboratory, was used to evaluate RR inhibition [1]. Exponentially growing cells (murine leukemia, P388) were incubated (at 37°C) in fresh medium (3×10^6 cells/mL, 4 mL/sample) with various concentrations of each compound. After 90 min, 0.32 μCi [^{14}C]cytidine was added to each sample (0.08 μCi/mL) for a further 30 min. These cells were, then, rapidly washed twice in ice-cold saline solution, put in Eppendorf tubes (10^7 cell/tube), disrupted with ice-cold 5% trichloroacetic acid for 20 min, and centrifuged (3000 × g, 4 min) at 4°C. Both the supernatant (acid soluble material) and pellet (nucleic acids) were kept and treated separately.

The supernatant was neutralized and processed as previously described by Steeper and Steuart [23] and subsequently modified by Cory *et al.* [24] to separate [^{14}C]deoxycytidine and its nucleotides from [^{14}C]cytidine and its nucleotides.

The pellet was resuspended in 800 μL of ice-cold 80 mm Tris(hydroxymethyl)aminomethane (pH 8) to which Dnase-free Rnase (250 μg/mL) had been added. The final pH was 7.8. After 2 hr of incubation at 37°C, the solution was cooled in ice and 100 μL 50% trichloroacetic acid were added. The solution was left for 15 min at 4°C and trichloroacetic acid-precipitable material was obtained by centrifuging (400 × g, 3 min, 4°C). After the addition of 15 mL of scintillation fluid (Insta-gel), the supernatant was transferred to vials and read in a beta counter to evaluate the RNA-incorporated [^{14}C]cytidine. The pellet was washed 4 times in ice-cold 5% trichloroacetic acid (500 × g, 3 min, 4°C) and dissolved in 400 μL of 1.25 M NaOH overnight. The sample was prepared for scintillation counting by mixing it with 19 mL scintillation fluid to evaluate DNA-incorporated [^{14}C]cytidine.

Ribonucleotide reductase activity was the sum of the [^{14}C]cytidine cpm incorporated into DNA and the [^{14}C]deoxycytidine (plus its nucleotides) cpm. For each concentration of compound tested, residual enzymatic activity was expressed as a percentage ratio of RR activity in the treated sample vs the nontreated control.

Data Analysis

The IC_{50} was calculated using Chou's median-effect equation [25], $f_i/(1 - f_i) = (C/IC_{50})^m$, where: C represents the concentration of drug that produces a determined fractional inhibition of the system, f_i represents inhibition value, and m the Hill-type coefficient. This coefficient indicates the degree of sigmoid shape in the dose-effect curve. If the correlation coefficient for the regression line is greater than 0.9, the equation can be considered to fit the dose-effect relationship. In our analyses, the value of the correlation coefficient was always greater than 0.95.

RESULTS

In vitro *Antitumor Activity*

In Table 1, the cytotoxic activity of BPYTA-Cu is compared with that of BPYTA and its ferrous complex. IC_{50} values were determined by 2 different schedules of cell-compound contact. Results shown indicate that BPYTA-Cu is the most active compound after pulse contact, but that it is less active than BPYTA after continuous contact. Using BPYTA and BPYTA-Fe, cell growth inhibition activity increased proportionally with increased time of contact. In the case of BPYTA-Cu, cell growth inhibition activity only doubled. This might suggest that BPYTA-Cu is highly reactive inside the cells and exerts its cytotoxic effect rapidly. Data concerning cytotoxic activity of BPYTA-Cu with prolonged contact may not be significant, considering the cytotoxic activity of copper salts.

Ability of BPYTA-Cu to Destroy the R2 Radical

To evaluate the effect of BPYTA-Cu on R2, we used recombinant mouse R2 obtained by the method of Mann *et al.* [21]. The R2 subunit of RR contains a tyrosyl free-radical that is required for enzyme activity, as suggested by EPR studies [1, 26]. Using this technique, it is possible to

TABLE 1. Antiproliferative activity in murine leukemia cell line P388 after 1 hr of compound-cell contact plus 65 hr recovery (pulse contact) or 66 hr of compound-cell contact (continuous contact)

	IC_{50}*	
	Pulse contact	**Continuous contact**
BPYTA	884.3	6.80
BPYTA-Fe	1435.6	28.03
BPYTA-Cu	59.46	24.87
$CuCl_2$	>2000	90.26

* Concentration (μM) inhibiting 50% [^{125}I]-5-iodo-2'-deoxyuridine incorporation in the cells. Results are the mean of at least 5 tests, separately analyzed.

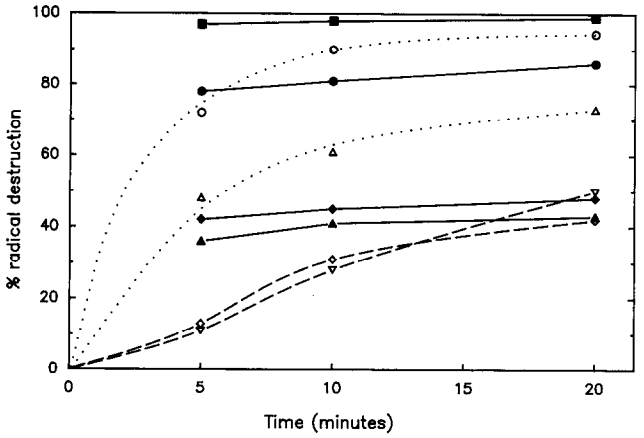

FIG. 1. Radical destruction of murine recombinant R2 samples treated with BPYTA and its metal complexes, after different times of contact at 37°C. Radical destruction was expressed as the % ratio between the height of the EPR peak spectrum recorded for the treated sample and that recorded for the control sample. The free radical concentration of the control sample was equal to 5.0–6.5 μM in the different experiments, but every thawing-freezing procedure caused a 10–15% decrease in signal. The line-linked symbols refer to values recorded for the same sample, thawed and treated for the required additional time; the obtained values were compared with the respective thawed-treated control sample. The solid line linking filled symbols refers to values obtained by treating with BPYTA-Cu: 200 μM alone (●) or in combination with 200 μM Fe(II) (■), 50 μM alone (♦), or in combination with 50 μM Fe(II) (▲). The dashed line refers to values obtained by treating with BPYTA alone: 200 μM (▽) or 50 μM (◇). The dotted line refers to values obtained by treating with BPYTA plus Fe(II) at a molar ratio of 1:1: 200 μM (○) or 50 μM (△).

avoid the presence of reducing substances or metal ions that may hamper the interpretation of results.

In Fig. 1, the relative radical destruction caused by BPYTA-Cu is compared with that of BPYTA alone or in the presence of Fe(II). With 5 min of compound-R2 contact, BPYTA-Cu was as active as BPYTA-Fe in destroying the tyrosyl radical. BPYTA, on the other hand, was inactive. This means that copper, like iron, renders BPYTA active. However, considering the time-course of radical inactivation, BPYTA-Cu activity differs from that of BPYTA-Fe. Using BPYTA-Fe, R2 inhibition increased with increases in concentration and/or time of contact, because the same inhibition was observed after 5-min contact with 200 μM and 20-min contact with 50 μM [1]. With BPYTA-Cu, R2 inhibition increased with increased concentration, but not with time of contact. Moreover, it is not dependent on iron concentration, even if a high iron concentration seemed to potentiate the radical destruction. In comparing BPYTA-Cu with BPYTA, it should be remembered that the relatively good inhibition caused by BPYTA after 10 or 20 min was not proportional to its concentration. In fact, it was correlated to the uncontrolled increase in iron concentration in the buffer during the time of contact, due to the release from R2, under the experimental conditions used [1]. Accordingly, after 20 min, BPYTA was as active as BPYTA-Cu, solely in relation to the uncontrolled iron.

Ribonucleotide Reductase is Not the Only Cell Target of BPYTA-Cu

To study whether or not the main BPYTA-Cu cell target is RR, two cell lines resistant to RR inhibitors were used. Figure 2 shows the lack of cross-resistance to BPYTA-Cu in a cell line made resistant to BPYTA [15] and in a cell line made resistant to HU [19]. These results suggest that RR is not the only target of BPYTA-Cu.

Study of [^{14}C]cytidine metabolism confirmed this hypothesis. With this method, the inhibition of RR activity and of RNA synthesis are simultaneously evaluated and the IC_{50}s are compared to those referring to cell growth inhibition obtained after 66 hr of compound-cell contact (Table 2). The ratio IC_{50} (antiproliferative activity)/IC_{50} (ribonucleotide reductase activity) was >1 for BPYTA, BPYTA-Fe, BPYTA-Cu, and HU, meaning that the concentration inhibiting cell growth is sufficient to inhibit RR. Consequently, RR can be considered a major intracellular target of each compound and the cell growth inhibition may be explained as a consequence of enzyme inhibition. However, BPYTA-Cu also inhibited RNA synthesis at concentrations inhibiting cell growth, and the IC_{50}s of RNA synthesis for BPYTA and BPYTA-Fe were far above their respective RR IC_{50}s. This means that RR is not the only major target of BPYTA-Cu.

The BPYTA-Cu(II) Complexes

Spectra obtained by dissolving BPYTA-Cu in buffers at pH 5 (Fig. 3, spectrum I) and pH 7.5 (Fig. 3, spectrum IV) were compared to those of BPYTA and BPYTA-Fe. Spectra obtained for BPYTA-Cu were unchanged a week after solubilization.

The formation of BPYTA-Cu spectrum was also studied

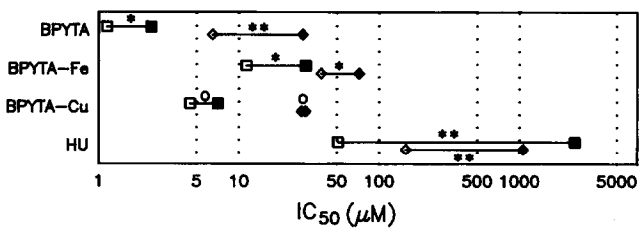

FIG. 2. Difference between the inhibitory concentration (IC_{50}) of cellular lines resistant to RR inhibitors (filled symbols) and the respective parental cellular lines (open symbols). ■, IC_{50} on TA3-H2 (cellular line made resistant to HU); □, IC_{50} on TA3 (parental cell line); ♦, IC_{50} on P388-R4 (cellular line made resistant to HU); ◇, IC_{50} on P388 (parental cell line). If $P < 0.01$ (**) or $P < 0.05$ (*), the resistant cellular line was significantly cross-resistant to the tested compound. If $P > 0.05$ (0) lack of cross-resistance was observed.

TABLE 2. Antiproliferative activity of the reported molecules compared to their inhibition of 2 cell pathways (ribonucleotide reductase activity and RNA synthesis)

	IC_{50}		
	Antiproliferative activity*	Ribonucleotide reductase inhibition†	RNA synthesis inhibition†
BPYTA	7.2	2.8	>64
BPYTA-Fe	29.5	23.3	>128
BPYTA-Cu	28.0	4.2	22.1
HU	312.1	36.8	>1000

The logarithmically growing tumor cell line P388 was used for each evaluation.

* Concentration (μM) inhibiting 50% [^{125}I]-5-iodo-2'-deoxyuridine incorporation in the cells. Results are the mean of at least 3 tests, separately analysed.

† Concentration (μM) inhibiting 50% [^{14}C]deoxycytidine synthesis (ribonucleotide reductase activity) or [^{14}C]cytidine incorporation in RNA (RNA synthesis). Results are the mean of 2 tests, separately analysed.

by adding copper to BPYTA in solution. In reducing condition (sodium dithionate solution) and at both pH values, the addition of Cu (I) salts caused the sudden appearance of a spectrum that can be explained as being the sum of the BPYTA spectrum, the sodium dithionate spectrum, and the Cu (I) salt spectrum (not shown).

In acetate buffer (pH 5) and a nonreducing condition, the addition of BPYTA to an equimolar concentration of Cu (II) salts gave an absorption spectrum qualitatively and quantitatively identical to that described by I (Fig. 3b). Lower concentrations of Cu (II), which resulted in higher BPYTA:Cu molar ratios (2:1, 3:1, and 4:1), resulted in a peak in the visible spectrum similar to, but less intense than, that described by I. Quantitative analysis of these results supports the hypothesis that the complex present in this solution is BPYTA-Cu(II) 1:1. On the contrary, in the

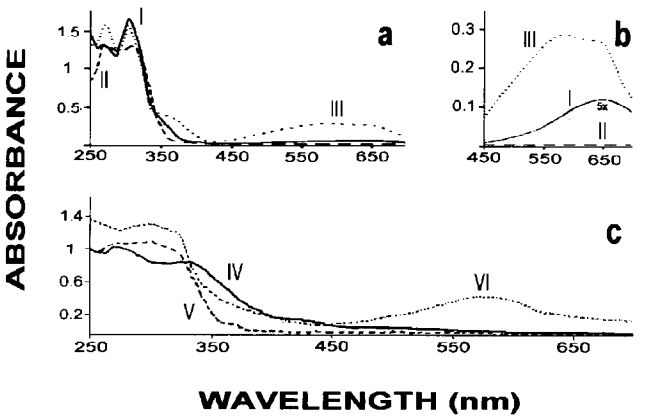

FIG. 3. UV-visible absorption spectra. a: 100 μM BPYTA-Cu (I, solid line), 100 μM BPYTA (II, dashed line), and 100 μM BPYTA-Fe (III, dotted line) in an acetate buffer (pH 5). b: The visible wavelength spectra for the above-specified conditions at a higher definition. In this Fig. (3b), the spectrum for 500 μM BPYTA-Cu is shown instead of 100 μM. c: 100 μM BPYTA-Cu (IV, solid line), 100 μM BPYTA (V dashed line), and 100 μM BPYTA-Fe (VI, dotted line) in a Tris-KCl buffer (pH 7.5).

Tris(hydroxymethyl)aminomethane KCl buffer (pH 7.5), the BPYTA-Cu (II) spectrum (IV) formed following copper salt addition resulted in a BPYTA-Cu (II) complex having a molar ratio of 2:1. When the pH was changed from 5 to 7.5 by adding sodium hydroxide, spectrum I rapidly changed to that of IV, and the passage from pH 7.5 to pH 5, achieved by adding hydrochloric acid, did not cause any modification of the spectrum. This suggests that the formation of the 2:1 complex is not favored at low pH but, when the BPYTA-Cu (II) 2:1 complex is formed, it is a stable complex.

BPYTA Complexes in the Presence of Cu (II) and Fe (II)

Because the activity and, perhaps, the mechanism of action of BPYTA heavily depends on the kind of metalloelement complexed with it, the tendency of BPYTA to form a complex was evaluated in the presence of Fe (II) and Cu (II). These studies were performed in acetate buffer (pH 5) to slow down the formation of Fe (III) from Fe (II), when iron was not complexed by BPYTA. The addition of Fe (II) to BPYTA-Cu (up to 50-fold excess) did not modify the BPYTA-Cu spectrum, but the addition of an equimolar concentration of Cu (II) to BPYTA-Fe caused the appearance of the BPYTA-Cu spectrum. The reaction was slow at room temperature but was complete after 15 min at 37°C. This suggests that the affinity of BPYTA towards Cu (II) is much higher than towards Fe (II).

HPLC analyses suggest that neither iron nor copper BPYTA complexes dissociate after dissolution in culture medium (Table 3). Moreover, they do not bond covalently with proteins present in the medium. This suggests that the concentration of iron and copper complexes may not be modified in plasma. However, the presence of available copper could favor the formation of BPYTA-Cu complexes instead of BPYTA-Fe complexes, because of the high BPYTA affinity for copper. This suggestion is supported by the cytotoxic activity of BPYTA in the presence of both iron and copper (Table 4). In fact, every time copper was present, the activity of BPYTA, free or complexed with iron, tended to be equal to that of BPYTA-Cu.

DISCUSSION

BPYTA-Cu is a potent inhibitor of tumor cell growth (Table 1) and should be of use in the treatment of cancer.

TABLE 3. Stability of BPYTA complexes after solubilization in RPMI-1640 medium supplemented with 15% heat-inactivated newborn calf serum and the other chemicals specified in Materials and Methods, as evaluated by HPLC-studies

	% as BPYTA*	% as BPYTA-Fe*	% as BPYTA-Cu*
BPYTA-Fe	7	93	0
BPYTA-Cu	0	15	84

* Percentage of the sample eluted at the time characteristic of the specified compound or complex.

TABLE 4. Antiproliferative activity in murine leukemia cell line P388 after 1 hr of compound-cell contact plus 65 hr recovery (pulse contact)

	IC_{50}*
BPYTA	791.6
BPYTA + $CuCl_2$	110.2
BPYTA + $FeSO_4$	1308.1
BPYTA + $CuCl_2$ + $FeSO_4$	103.8
BPYTA-Cu	55.3
BPYTA-Cu + $FeSO_4$	80.8
BPYTA-Fe	1472.9
BPYTA-Fe + $CuCl_2$	135.5
$CuCl_2$	>2000
$FeSO_4$	>4000

Compounds were tested alone or in association with equimolar concentrations of salts.

* Concentration (μM) inhibiting 50% [^{125}I]-5-iodo-2′-deoxyuridine incorporation in the cells. Results refer to a representative experiment.

The R2 subunit of RR is a cellular target of BPYTA-Cu (Fig. 1), but it is likely that the inhibition of other cellular targets is equally responsible for the cytotoxic activity of BPYTA-Cu.

BPYTA has been demonstrated to have certain properties in common with α-HCATs [1] and the characteristic of the copper complex confirms the similarity. Saryan *et al.* [8] suggested that, after a pulse contact, α-HCAT metal complexes were present in higher concentration in cells, with respect to the ligand, and that only the metal complexes had cytotoxic activity. This is also what seems to happen with BPYTA-Cu, which is more cytotoxic than BPYTA, after a pulse contact. Spectrophotometric and HPLC studies (Fig. 3 and Table 3, respectively) demonstrate that the copper complex of BPYTA is a 2:1 BPYTA-Cu (II) complex that is stable in solution. α-HCATs also chelate Cu (II), but at a molar ratio of 1:1 [27].

Despite the fact that the mechanism of action of α-HCATs is widely recognized as being that of RR inhibitors [3], some authors have hypothesized that their copper complexes could have different mechanisms of action [11, 12, 28]. In particular, Byrnes *et al.* have hypothesized that thiol oxidation, itself, may prove to be the source of copper-thiosemicarbazone toxicity [10]. Even if our data are not sufficient to establish this hypothesis for BPYTA-Cu, they are certainly consistent with all of the reported results. In fact, it may be that the powerful cytotoxic activity of BPYTA-Cu after a short period of contact (Table 1) is due to the reaction with cell proteins containing -SH groups that do not release the complex during cell washings. RR inhibition by BPYTA-Cu may be due more to the reaction with enzyme -SH groups than to free radical destruction. This could explain the lack of time-dependence observed in the R2 study (Fig. 1). RNA synthesis inhibition could also be the consequence of BPYTA-Cu reaction with other cellular proteins.

Differences observed when BPYTA chelates with copper or iron are notable. BPYTA-Cu seems to cross cellular membrane freely because 1 hr of cell-complex contact is sufficient to powerfully inhibit cell growth (Table 1). On the contrary, cellular uptake for BPYTA-Fe seems to be very slow [1]. The R2 radical destruction caused by BPYTA-Fe was reversible and proportional to time of contact and to concentration [1], but the R2 radical destruction caused by BPYTA-Cu was independent of time of contact (Fig. 1). This suggests that BPYTA-Cu reacts rapidly with the target and that the complex may be inactivated-diassociated during the reaction. The most intriguing finding, however, is that RR is only one of the cellular targets of BPYTA-Cu (Fig. 2 and Table 2), and RR is the main cellular target of BPYTA-Fe [1]. At the present time, it is difficult to hypothesize whether these differences in mechanism of action depend on the different oxidation state of the metalloelement chelated by BPYTA (iron is chelated by BPYTA in the reduced form and copper in the oxidized form) or on the different properties of iron and copper.

In conclusion, the antiproliferative effects of BPYTA-Cu seem to be less target-specific than those of BPYTA-Fe. Because the affinity of BPYTA towards copper is much higher than that towards iron, it seems likely that the activity of BPYTA-Fe on different cellular targets (less important for cytotoxicity than R2 targeting) [1] is due to the dissociation of some BPYTA-Fe complexes and the formation of BPYTA-Cu. Moreover, it may be the case that the mechanism of action of BPYTA (inactive by itself) depends on the concentrations of metalloelements readily available from cellular stores.

The authors wish to express their gratitude to Prof. Astrid Gräslund and Prof. Lars Thelander, Department of Medical Biochemistry, Biophysics, University of Umeå, Sweden, for providing tecnical facilities and Göran Wikander, Department of Physical Chemistry, University of Umeå, Sweden, for access to EPR facilities. G.N. wishes to thank Marghereta Thelander for excellent technical assistance during work at the University of Umeå. The authors wish to thank Prof. Palmarisa Franchetti, Department of Chemical Sciences, University of Camerino, Italy, for supplying BPYTA and its metal complexes, Prof. Stefano Rufini for performing HPLC studies, and Federica Federici for expert technical assistance. The work was supported in part by the Ministero della Università e della Ricerca Scientifica e Tecnologica, Rome (Italy).

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