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Isolation of two cellular lines resistant to ribonucleotide reductase inhibitors to investigate the inhibitory activity of 2,2′-bipyridyl-6-carbothioamide

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The mechanism of action of a synthetic compound—2,2′-bipyridyl-6-carbothioamide—was investigated by developing tumor lines resistant to it (P388-R1.5 and P388-R4). P388-R4 is resistant to inhibitors of ribonucleotide reductase (RR) while no resistance was observed to antitumor drugs having other targets (except to bleomycin). The resistance to inhibitors of the RR M_2 subunit is higher than that to compounds active on the RR M_1 subunit. Moreover, murine chromosome 12, in which the M_2 structural gene was recently localized, was trisomic in the resistant lines. We conclude that it is possible to consider BPYTA a new inhibitor of the RR M_2 subunit.

Key words: 2,2′-bipyridyl derivative, chromosome 12 trisomy, *in vitro* study, P388 resistant line, ribonucleotide reductase inhibitors.

Introduction

2,2′-bipyridyl-6-carbothioamide (BPYTA)[1] is characterized by the N*–N*–S* tridentate ligand system and has structural analogies with α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones (α-HCAT), potent inhibitors of ribonucleotide reductase (RR).[2] This enzyme[3] is responsible for converting ribonucleoside diphosphates to the deoxyribonucleotide precursors of DNA; the reaction is rate limiting. RR has unusual structural and regulatory properties, and plays a key role in the biology of the normal and neoplastic cell. It consists of two non-identical subunits often called M_1 and M_2,[4] both of which are required for its activity.[3] Hydroxyurea (HU) is the only RR inhibitor used successfully in some antitumor treatments;[5,6] however, it is not a potent drug and it seems important to design other RR inhibitors.

The *in vitro* evaluation of the BPYTA antitumor spectrum,[7] its activity in association with chelating molecules (submitted manuscript) and the cytotoxic activity of its metal complexes,[8,9] enabled us to hypothesize that BPYTA is an RR inhibitor. In order to verify whether RR is the target of BPYTA, and particularly the main target, we developed sublines of the murine leukemic cell line P388 resistant to BPYTA. A panel of molecules with a known mechanism of action was tested on the cell lines. Cytogenetic analysis of the resistant lines was also performed.

Materials and methods

Tumor cell line and media

The murine leukemic line adapted to *in vitro* growth, P388, was maintained in continuous

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suspension culture in RPMI-1640 medium at pH 7.2 containing antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, gentamicin 50 μg/ml), and supplemented with 15% fetal calf serum, 3 mM glutamine, 10 mM HEPES buffer and 0.01 μM 2-mercaptoethanol. Cells were grown exponentially in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

RPMI-1640, fetal calf serum and additive solutions were purchased from Biochrom-Seromed, Berlin, Germany. [^{125}I]-5-iodo-2′-deoxyuridine (^{125}IUdR), 35.4 Ci/mmol, was obtained from Amersham, England.

Generation of P388-R1.5 and P388-R4 cell lines

The P388 cell line (P388-S) was initially treated in two different ways.

(1) It was maintained in continuous compound-cells contact with increasing concentrations of BPYTA; the treatment was started at 4 μM and increased weekly by 5–10%.
(2) It was treated with a pulse contact (24 h) of BPYTA plus recovery. The treatment was repeated every 10–12 days, the BPYTA concentration being increased by 20% each time (starting from 8 μM).

Treatment 1 was stopped at 10 μM, because cells did not grow any more. At that time significant resistance was not observed. The cell line subjected to treatment 2 reached about 1.5-fold resistance when treated with 50 μM BPYTA (P388-R1.5). The further increase of compound concentration to 200 μM did not change the resistance index.

In order to increase the resistance we set up a third protocol in which cells (previously treated with the second protocol, i.e. P388-R1.5) were maintained in continuous contact with 10 μM BPYTA. Cells were washed weekly by centrifugation and resuspended in a fresh medium to which the same BPYTA concentration was added. After 4 months of treatment the cell line reached about 4-fold resistance (P388-R4).

Compounds

BPYTA and 2-formylpyridine thiosemicarbazone (PT) were synthesized as previously reported.[1,9,10] 2-chloro-2′-deoxyadenosine (CdA) was synthesized by Franchetti as reported by Plunkett *et al.*[11] HU, deferoxamine (DF), cytarabine (AraC), daunorubicin (DNR), bleomycin (BLM), lomustine (CCNU), vincristine (VCR) and 5-fluorouracil (5-FU) were respectively obtained from the commercial preparations Oncocarbide (Simes), Desferal (Ciba-Geigy), Aracytin (Upjohn), Daunoblastina (Farmitalia), Bleomicina (Nippon Kayaku), Belustine (Roger Bellon), Oncovin (Lilly) and Fluoro-uracile (Roche).

Evaluation of drug resistance

The antiproliferative assay developed for predictive evaluation of tumor chemosensitivity[12] has been previously used in *in vitro* studies on the antitumor activity of BPYTA.[9]

Briefly, various concentrations of each drug were placed with tumor cell suspension (1.5×10^5 cells/ml). 48 h later DNA synthesis was evaluated by adding 0.1 μCi/well of ^{125}IUdR along with 2′-deoxy-5-fluorouridine (0.01 μg/well) to the cultured cells for an additional 18 h. During this time the cells were still growing exponentially. Harvesting was performed by a multiple suction filtration apparatus (Mash II) on a fiberglass filter (Whittaker Co., USA). Paper disks containing the aspirate cells were read in a gamma scintillation counter.

Results are expressed as percentage ratio of inhibited radioisotope incorporation in the treated cultures vs. untreated controls. The median effect concentration that is required for 50% cytotoxicity (IC_{50}) was calculated using the median effect equation derived by Chou.[13] The ratio IC_{50}(P388-R)/IC_{50}(P388-S) was considered the resistance index. Reported IC_{50} were the mean of at least four different experiments, separately analyzed. The *P* values were calculated by Student's *t*-test.

Cytogenetic analysis

For karyotypic analysis, P388-R1.5 and P388-R4 were maintained in a BPYTA-free medium for a week. Cells were incubated with 0.1 μg/ml colchicine for 1 h at room temperature, treated with 0.075 M KCl solution for 10 min and fixed three times with methanolacetic acid (3:1). Air-dried preparations were stained using the G-banding technique according to Wang and Fedoroff;[14] the concentration of trypsin and the digestion were slightly modified.

Results

Resistance pattern

P388-R1.5 and P388-R4 were sequentially selected in the presence of increasing concentration of BPYTA for the ability to proliferate in normally cytotoxic compound concentrations as described in "Materials and methods". The acquired resistance was evaluated using an antiproliferative assay. Before testing, cell lines were maintained in a BPYTA-free medium for a week.

P388-R1.5 showed a resistance to BPYTA of 1.54-fold (IC_{50}: 10.99 ± 1.1 vs. 7.12 ± 0.61; $p < 0.05$) and to HU of 1.67-fold (IC_{50}: 278.712 ± 23.1 vs. 166.63 ± 14.7; $p < 0.01$); P388-R4 showed a resistance to BPYTA of 3.78-fold (IC_{50}: 28.49 ± 2.86 vs. 7.53 ± 0.76; $p < 0.01$).

In Figure 1 resistance indices of P388-R4 to inhibitors of RR M_1 and M_2 subunits with respect to BPYTA are reported. Previous studies have indicated that α-HCAT (as PT) and chelators (as DF) may have different mechanisms of inhibition of RR compared with HU even though they are all directed at the M_2 subunit.[15] Tests on P388-R4 have demonstrated that the molecules active on this subunit have the same resistance values as BPYTA, even if their mechanism of action differs. Resistance to CdA (active on RR M_1 subunit) and to AraC (weakly active also on RR M_1 subunit) was less.

The P388-R4 IC_{50} values of drugs that do not inhibit RR are similar to P388-S IC_{50} values except for DNR and BLM (Figure 2).

The whole pattern of resistance was stable and did not change during 1 month of continuous culture in the absence of BPYTA for both resistant lines.

Cytogenetic analysis

Karyotypic analysis showed clearly that the resistant cell lines have acquired one more chromosome (Figure 3). The modal chromosome number of parental and resistant cell lines was 39 and 40 respectively; the mean chromosome number was 38.96 for P388-S, 39.75 for P388-R1.5 and 39.62 for P388-R4.

In Table 1 a detailed study on the spread metaphases is reported. It demonstrated that the

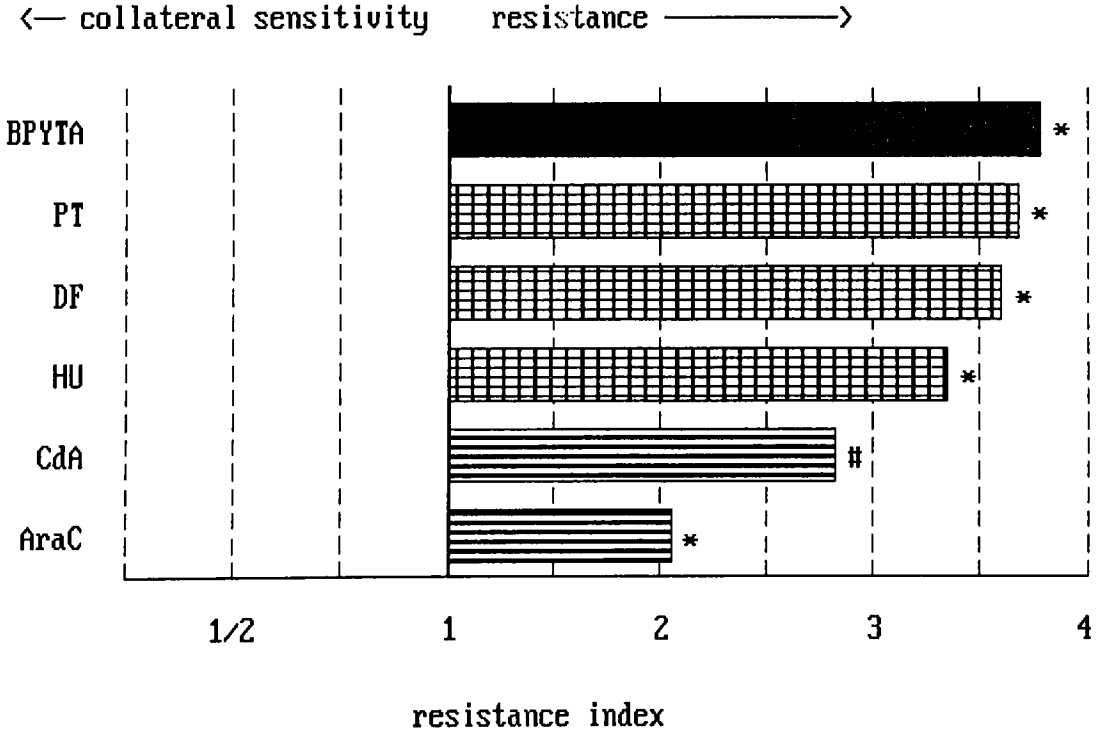

Figure 1. Ratio of the IC_{50} values on P388-R4 vs. P388-S for each drug tested, active on RR (reticulated bars, M_2 subunit; striped bars, M_1 subunit). * indicate $p < 0.01$ and # $p < 0.05$. IC_{50} values (μM ± 1 standard error) on P388-S were for BPYTA 7.53 ± 0.78, for PT 1.13 ± 0.31, for DF 19.58 ± 4.21, for HU 147.30 ± 14.7, for CdA 0.3131 ± 0.048, for AraC 0.080 ± 0.0029.

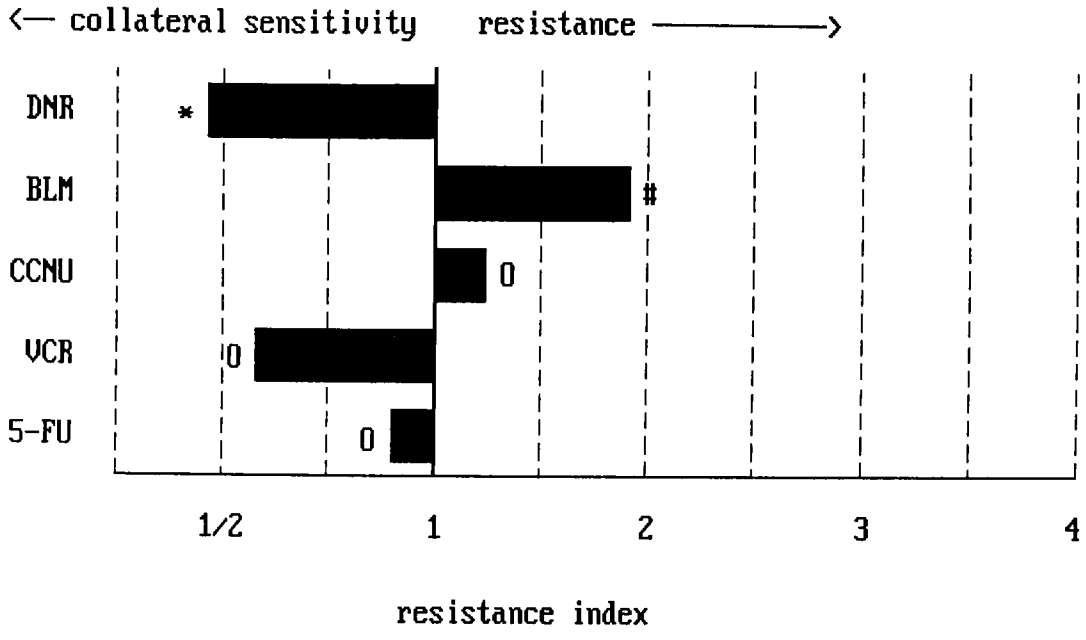

Figure 2. Ratio of the IC_{50} values on P388-R4 vs. P388-S for each drug tested, not active on RR. * indicates $p < 0.01$, # $p < 0.05$ and O $p > 0.05$. IC_{50} values ($\mu M \pm 1$ standard error) on P388-S were for DNR 0.1245 ± 0.021, for BLM 665.4 ± 104.3, for CCNU 58.02 ± 24.22, for VCR 0.0072 ± 0.0024, for 5-FU 1.022 ± 0.1383.

main cell population (i.e. the most frequently observed cell subpopulation) of P388-R1.5 and of P388-R4 cell lines has acquired a stable trisomy of chromosome 12 (Figure 4). None of the P388-S cells examined presented this kind of alteration. Other

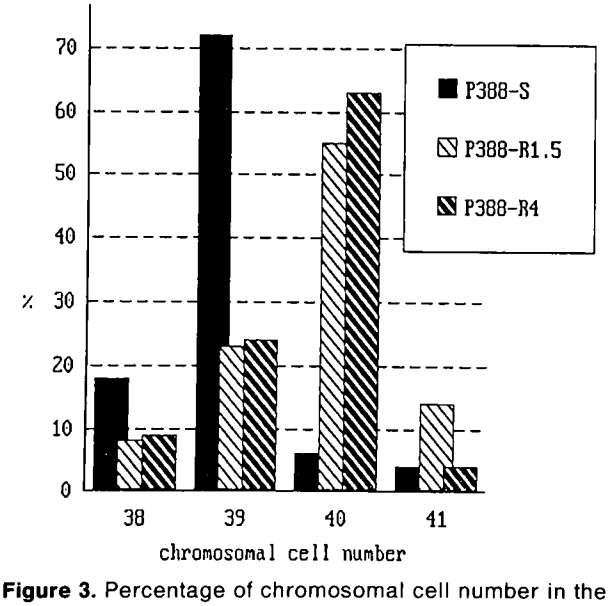

Figure 3. Percentage of chromosomal cell number in the parental and resistant cell line populations. 100 cells were counted for each cell population. The few cells with chromosome cell number equal to 37 were reported as 38 and the very few cells with chromosome cell number equal to 42 or 43 were reported as 41.

significant variations in chromosomal content and chromosomal marker were not observed in both cell lines (data not reported) and this supports the importance of this non-random change.

Chromosomal analysis of P388-R4 during the BPYTA treatment showed the lack of BPYTA clastogenic activity (chromosome breaking). Effects like those frequently caused by the formation of intermediate active oxygen species were observed.

Despite the different resistance indices, P388-R1.5 and P388-R4 have extremely similar cytogenetic characteristics.

Doubling time

Doubling time of P388-S and P388-R4 was evaluated when cellular concentrations ranged between 10^3 and 5×10^5. Before evaluating, P388-R4 was maintained in a BPYTA-free medium for two weeks. The mean doubling time was 13.9 ± 1.2 h for P388-S and 18.0 ± 0.9 h for P388-R4 ($p < 0.05$).

Discussion

Structural analogies of BPYTA with α-HCAT and its cytotoxic activity had suggested that this

Table 1. Percentage of cell showing trisomy of chromosome 12 in P388-S and P388-R cell lines as a function of chromosomal cell number

Chromosomal cell number	P388-S		P388-R1.5		P388-R4	
	f (%)[a]	*t* (%)[b]	*f* (%)[a]	*t* (%)[b]	*f* (%)[a]	*t* (%)[b]
39	72	0	23	40	24	20
40	6	0	55	78[c]	63	100
41	4	0	14	100	4	100

[a] Frequency of cells with the reported chromosomal number in each cell population.
[b] Cells showing trisomy of chromosome 12 in each cell subpopulation characterized by a specified chromosome cell number. Value is referred to nine, five or three spread metaphases studied as a function of the frequency of cells with the reported chromosomal number (>50%, between 50% and 15%, or <15% respectively).
[c] Of the nine metaphases analyzed, seven had trisomy and two tetrasomy of chromosome 12.

compound inhibits RR.[7–9] On the other hand, the chelating activity of BPYTA and its chemical structure left open other hypotheses regarding its mechanism of action. Having obtained cellular lines resistant to BPYTA which also demonstrate a significant cross-resistance exclusively to inhibitors of RR, strongly suggests that BPYTA exerts its cytotoxic activity by inhibiting RR.

In addition, the resistance index of BPYTA tested on P388-R4 is similar to that of M_2 inhibitors (HU, DF, PT) in comparison with the lesser resistance index of RR M_1 inhibitors. This suggests the hypothesis that our compound is active on the M_2 subunit.

Also, cytogenetic analysis supports this conclusion. Recent studies indicate that, in human and rodent cells, the genes for RR M_1 and M_2 subunits are located on different chromosomes[16,17] and in particular the structural gene of the RR M_2 subunit is localized in the chromosome 12 of the murine genome.[17] The trisomy of chromosome 12 observed in the main part of the cell population of the P388-R cell lines could be responsible for M_2 overproduction and could explain the resistance to M_2

Figure 4. G-banded metaphase plate of P388-R4. Note the presence of trisomy 12 (arrows).

inhibitors (BPYTA included). In fact any chromosomal trisomy of mouse cells has been demonstrated to cause a significant cellular increase of a small part of the proteins specifically coded by the trisomic chromosome genes.[18,19] To date the only experimental datum confirming our hypothesis is that the resistance indices are identical for molecules acting with different mechanisms on the same target (M_2). So it is likely that resistance is not due to any target modification.

It should be noted, however, that the role of the trisomy in the mechanism of acquired resistance to BPYTA is probably important only at the first step of resistance induction. In fact, on increasing resistance, the polysomy of chromosome 12 did not increase. On the contrary, a little subclone showing tetrasomy of chromosome 12 observed in P388-R1.5 was missing in P388-R4.

Moreover, the time necessary for the synthesis of one more chromosome could in part explain the slight increase of P388-R4 doubling time. Otherwise the acquired resistance might be a consequence of the increased doubling time through the modification of some feedback systems during the S phase.

Resistance to CdA and to AraC can be attributed, in accordance with the above-reported hypothesis on M_2 overproduction, to the increased probability that the M_1 subunit forms the active complex with the M_2 subunit. However, the explanation for the resistance to AraC could be more complex and could involve its main mechanism of action (DNA polymerase inhibition). If there were an expansion of deoxyribonucleotide pools in the resistant cellular line, the intracellular activated AraC could be diluted and could exert its cytotoxic activity only at higher concentrations.[20]

The different sensitivities of P388-R4 to DNR and BLM with respect to P388-S could also suggest that this cell line has modified in some way the uptake and/or the intracellular distribution of ferrous ions. It is known that both drugs carry out their activity on DNA using a ferrous ion and BLM was recently found to be a potent RR inhibitor, following brief exposure to HU, through its metal-chelating properties.[21] Moreover, α-HCAT and BPYTA have strong links with ferrous ions (2), (8), (9). Attempts to verify the hypothesis are in progress. Moreover, working with metal ions on cellular systems involves the possibility of obtaining contradictory results that are not easily interpreted.[22,23]

It is difficult to hypothesize why the induction of resistance was so long and difficult and why the resistance obtained is relatively low. It could be said that treatment 2 (pulse contact) was necessary for the selection of a cellular clone potentially capable of developing a good resistance; however, only the treatment with relatively low BPYTA concentrations was able to increase resistance (induction?). That seems to be an empirical confirmation of the studies on the crucial importance of RR for the fidelity of DNA replication. Alteration in RR activity (spontaneous or caused by non-cytocidal drug concentrations) are accompanied by increased rates of spontaneous mutation due to an imbalance of endogenous deoxyribonucleoside triphosphate pools.[24,25]

As far as we know there are several cell lines which have been made resistant to RR inhibitors,[20,26,27] but none of these was obtained using compounds active on the RR M_2 subunit with a mechanism of action different from HU. This fact might signify that there are intrinsic difficulties in developing resistance to this group of compounds. Moreover, it should be noted that the cellular lines resistant to HU do not have significant cross-resistance to α-HCAT,[26] while our cell line is equally resistant to all inhibitors directed at the RR M_2 subunit. That could signify that the mechanism of resistance is original and completely separate from the specific mechanism of action. So this cell line could be an interesting tool in studying RR inhibitors. In our laboratories the inhibitory activity of some of these compounds on P388-S RR and on P388-R4 RR is being tested, through the evaluation of the enzyme activity and its inhibition on an intact cell system.

Conclusion

Results presented suggest it is possible to consider BPYTA a new inhibitor of the RR M_2 subunit. Since BPYTA seems to have a different mechanism of action with respect to α-HCAT (submitted manuscript), this molecule and its analogs could represent a new class of RR M_2 inhibitors.

The cell lines isolated have some original characteristics and could be interesting tools in studying RR inhibitors.

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