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Potentiation of the Antitumour Activity of 2-Formylpyridine Thiosemicarbazone by Metal Chelation: 2-Formylpyridine Thiosemicarbazone Zinc Sulphate (NSC 294721)*

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Abstract—*A* Zn *chelate of* 2-*formylpyridine-thiosemicarbazone* (2-*FPTS*-Zn) *has been synthetized and its antitumour activity has been investigated on a spectrum of tumours transplantable in mice: the ascitic form of* L1210 *lymphoid leukemia, the ascitic and blood forms of* P388 *lymphocytic leukemia and solid tumours such as Melanoma* B16 *and Lewis lung carcinoma. The therapeutic activity of the chelate was compared to that of the free drug against leukemias. The following activities were established.*

The 2-*FPTS*-Zn *chelate showed a higher therapeutic activity against leukemias as compared to the free drug. Treatment with the chelate induced a high percentage of increase in life span* (151–157%) *of* L1210 *leukemia bearing mice over controls and a high percentage of long-term survivals in treated groups* (10–40%) *was recorded when the optimal schedule of treatment was used* (6–8 mg/kg *given every* 3 hr *on* days 1, 5 *and* 9 *after tumour cells inoculation*).

The Zn *chelate significantly inhibited the formation of lung metastases in the Lewis lung tumour and was inactive against the* B16 *tumour under the conditions of the experiment. A good direct contact between the chelate and the tumour cells seemed to be required to induce therapeutic activity.*

INTRODUCTION

THE ANTINEOPLASTIC activity of various carbazone of α-(*N*)-heterocyclic carboxaldehydes has been investigated [1–6]. Several of these derivatives like 1-formyl isoquinoline thiosemicarbazone [1, 2], its 5-hydroxy derivative [3] and both 3-hydroxy-2-formylpyridine thiosemicarbazone and 5-hydroxy-2-formylpyridine thiosemicarbazone [4, 5] showed a pronounced antineoplastic activity when tested on a relatively wide spectrum of tumours transplanted in mice and finally, a 4-substituted 2-formylpyridine thiosemicarbazone, the morpholino-2-formylpyridine thiosemicarbazone, showed a very high antineoplastic activity against ascitic Sarcoma 180 [6]. Heterocyclic thiosemicarbazones showed not only antitumour but also antiviral activity [7].

These α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones primarily block the DNA synthesis in mammalian cells by inhibiting the enzyme ribonucleoside-diphosphate reductase [7, 8], which is necessary to transform nucleoside diphosphate into deoxynucleoside diphosphate.

The 5-hydroxy-2-formylpyridine thiosemicarbazone is the only drug in this series that has been administered to humans as part of a phase I study [9, 10].

However, the impressive antineoplastic activity displayed by this drug when used on animal models was not yielded when used on humans and this, apparently, because of its

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rapid inactivation and excretion [9]. An anti-leukemic effect of the drug was demonstrated in a few studies [9–11] but dose limiting gastro-intestinal toxicity as well as other toxicities including myelosuppression and hemolysis were found [10, 11]. A dark green urine reflected an increased excretion of chelated iron. Therapeutic doses were too close to toxic doses.

Of all series which were synthetized, one of the most promising seems to be the 2-formylpyridine-thiosemicarbazone (FPTS—Fig. 1). Yet, the FPTS belongs to the relatively water insoluble and toxic group: the activity level is only reached at a toxic level.

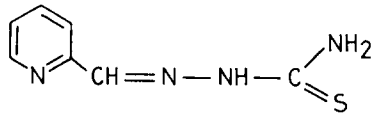

Fig. 1. 2-Formylpyridine thiosemicarbazone.

On the other hand, it is known that Zn salts such as sulfate and chloride are useful in the treatment of small skin carcinomas and ulcerations. It was also reported that Zn acetate prevented tumour growth in mice [12].

The FPTS is able to form two coordination compounds with divalent ions and, particularly, with Zn, iron and copper. The typical Zinc chelate, well known by the analytical chemists, is formed in alkaline media and appears as a heavy bright yellow precipitate almost insoluble in all solvents except in acids. Another chelate can be formed in acidic solution and appears as a pale yellow precipitate finely divided and soluble in water.

Some authors reported on metal complexes of FPTS [13] but those complexes are different from the one we have used. Therefore, in an attempt to find a less toxic agent and a water soluble compound for parenteral administration, a series of metallic chelates of 2-formylpyridine thiosemicarbazone was synthetized and, in a first step, the potential antitumour effectiveness against murine tumour of the soluble Zn chelate (FPTS-Zn—Fig. 2) was investigated and compared to that of the free drug against leukemias. The results of these studies are given below.

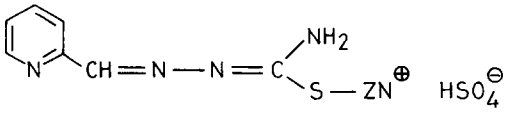

Fig. 2. 2-Formylpyridine thiosemicarbazone zinc sulphate.

MATERIALS AND METHODS

Drugs

Preparation of the 2-formylpyridine thiosemicarbazone Zn *sulphate coordination compound.* The 2-formylpyridine thiosemicarbazone reacts completely with Zn ion in solution. The 1:1 complex is formed by the reaction of 20 g of Zn $SO_4 . 7H_2O$ dissolved in 200 ml of distilled water and 10 g of slowly added ligands. Stirring gave a limpid solution in about 15 min. Stirring was continued for 45 min and the Zn chelate precipitated. After a one night rest in an icy box, the Zn complex was purified by repeated washings in icy water and then by recrystallization. Chemical analyses were performed. The ligand was determined by spectrophotometry: E (1%/1) cm = 520 at 350 nm. Quantitative determination of Zn was performed after ignition and titration with EDTA. The comparison of the i.r. spectrum of the ligand with that of the Zn complex proved the existence of the complex. This analysis of the product is consistent for a 1:1 complex in solid state.

The resulting complex showed the following i.r.-spectrum (in KBr): maxima at 1665, 1630, 1240, 1180, 1065 and 750 cm^{-1}.

The drug was dissolved in saline before administration to animals.

The free drug. The free drug 2-formylpyridine thiosemicarbazone was offered by Recherches Biochimiques et Pharmacologiques, Brussels. The drug was suspended in saline.

Animals

BDF_1 (DBA/2 × C57 Bl/6), DBA/2 and C57 Bl/6 mice (supplied by Charles River Breeding Laboratories) were used.

Tumours

The P388 lymphocytic leukemia, L1210 lymphoid leukemia, Melanotic melanoma B16 and Lewis lung carcinoma were originally obtained from I. Wodinsky (Arthur D. Little, Inc., Cambridge, Mass.). These tumours are maintained in our laboratory by serial transfer in mice.

1. *P388 lymphocytic leukemia.* P388 ascites were aspirated from DBA/2 mice and a suspension was made in saline; 10^6 cells of 0.1 ml solution were inoculated intraperitoneally (i.p.) in BDF_1 mice weighing 22–27 g to obtain the ascitic form of leukemia and 10^5 cells were inoculated intravenously (i.v.) in

mice weighing 21–24 g to obtain the blood form.

Animals were randomized into test and control groups as in all experimental tumour models that follow.

Treatment was started 24 hr after inoculation of the leukemic cells and administered i.p. once a day for 9 days, in the case of ascitic leukemia and i.v. once a day for 4 days, in the case of the blood form leukemia.

The increase in life span (I.L.S.) expressed as a percentage was taken as a parameter of therapeutic activity.

$$\text{I.L.S.}\% = \frac{\text{median survival time of treated group} \times 100}{\text{median survival time of control group}} - 100$$

The same parameter was used in all the tumour systems below.

2. *L*1210 *lymphoid leukemia.* A hundred thousand cells, taken from DBA/2 mice, were injected i.p. to BDF_1 mice weighing 23–26 g.

Treatment started 24 hr after implantation of the leukemic cells and was administered i.p., in one experiment, and orally (p.o.), in a second. The influence of the schedule of treatment was investigated in a third experiment. The route and timing of treatment are specified in each table.

3. *Melanotic melanoma B*16. A half of a millilitre of tumour suspension, from an homogenate of 1 g tumour (taken from C57 Bl/6 mice) in 9 ml saline, was implanted subcutaneously (s.c.) in BDF_1 mice weighing 20–25 g).

Treatment was started 24 hr after tumour transplantation and continued once a day for 9 days.

4. *Lewis lung carcinoma.* The tumour was excised from C57Bl/6 mice and an homogenate was made. BDF_1 mice weighing 22–27 g were inoculated intramuscularly (i.m.) in the left posterior leg with 2×10^6 cells.

Treatment was started i.p. 24 hr after tumour implantation and continued once a day for 15 days. Mice were randomized into test and control groups. Test and control groups were duplicated. The first half was intended for survival time evaluation. The second, intended for determination of tumour and metastases inhibition, was sacrificed 24 days after tumour cells inoculation. The tumours were then excised and weighed. Lung metastases were counted, their diameter was measured in mm and this measure was converted into weight (mg) according to the following formula: $W = r^3 . \pi\ 4/3$ (r = radius of the sphere in mm and W = weight in mg). It was assumed that a metastasis had the shape of a sphere and that its specific weight was equal to one.

RESULTS

1. *Antileukemic effect of 2-formylpyridine thiosemicarbazone* Zn *coordination compound as compared to the free drug and the influence of the schedule and route of treatment on this effect*

This study was performed on animals bearing P388 and L1210 leukemias. The number of animals in test and control groups is indicated in the tables.

Survival was significantly prolonged in animals bearing P388 leukemia and treated by the free form of 2-formylpyridine thiosemicarbazone (FPTS) or by 2-formylpyridine thiosemicarbazone zinc coordination compound (FPTS-Zn) (Table 1). The same table shows that the Zn chelate of 2-formylpyridine

Table 1. *Therapeutic effect of 2-formylpyridine thiosemicarbazone* (2-*FPTS*) *and its* Zn *chelate* (2-*FPTS*-Zn) *administered i.p. on the life span of* BDF_1 *mice bearing P*388 *ascitic leukemia*

	2-FPTS			2-FPTS-Zn		
mg/kg/injection	B.W.C. (g)	Med.S.T. (days)	I.L.S. (%)	B.W.C. (g)	Med.S.T. (days)	I.L.S. (%)
15.0	−4.3	11	10	−4.3	8	—
12.5	−1.4	16	60	−3.7	19	90
7.5	−0.5	16	60	−1.3	18.5	85
6.25	−0.1	15	50		15.5	55
3.12	+0.1	15	50		15	50
Controls	+1.6	10		+1.6	10	

BDF_1 mice were inoculated i.p. with 10^6 leukemic cells on day 0. Treatment was started 24 hr after tumour inoculation and continued for 9 days (one injection/day).
B.W.C. = body weight change on day 5.
Med.S.T. = median survival time of 8 treated mice and 36 untreated control mice.
I.L.S. = increase in lifespan of treated groups as compared to control groups.

thiosemicarbazone was therapeutically more active than the free drug. The 12.5 and 7.5 mg/kg doses of the free drug given once a day from day 1 to day 9 after tumour cells inoculation induced a 60% increase in life span (I.L.S.), while 12.5 and 7.5 mg/kg of the Zn chelate (corresponding to 6.6 and 3.95 mg/kg of 2-formylpyridine thiosemicarbazone since the chelate contains only 52.7% of the free drug), at the same schedule, induced a 90 and 85% I.L.S. respectively. An enhanced therapeutic effectiveness was also apparent with the blood form of P388 leukemia (Table 2) where 20 mg/kg of FPTS-Zn, given for 4 days after tumour transplantation, increased by 30% the survival time of leukemic mice over controls while the free drug was toxic at the same dose. The two other doses of the two drugs were not toxic and inactive under the conditions of the experiment.

In the case of L1210 ascitic leukemia, the survival time of the animals treated with Zn-chelate once a day during 9 days after tumour transplantation was longer than that of the mice treated with the free drug: at 12.5 mg/kg/day, the I.L.S. per cent induced by the chelate and the free drug were 76 and 43 respectively (Table 3).

Using the L1210 leukemia system, the influence of the schedule and route of treatment on the therapeutic activity was investigated. Tables 4 and 5 show that when the optimal schedule and route of treatment for the chelate and the free drug (every 3 hr on days 1, 5 and 9 after tumour cells inoculation) as well as the optimal doses (6.25 mg/kg × 8 for the chelate and 6 mg/kg × 8 for the free drug) were used, the therapeutic activity of the Zn-chelate was considerably superior to that of the free drug: the Zn chelate induced a 151% I.L.S. with 4 out of 10 mice surviving to day 50, while the free drug induced only a 89% I.L.S. with no long-term survivors.

Table 2. Therapeutic effect of 2-FPTS and its Zn chelate (2-FPTS-Zn) administered i.v. on the life span of BDF_1 mice bearing the blood form of P388 leukemia

	2-FPTS			2-FPTS-Zn		
Doses mg/kg	B.W.C. (g)	Med.S.T. (days)	I.L.S. (%)	B.W.C. (g)	Med.S.T. (days)	I.L.S. (%)
20	−5.8	10	—	−5.9	17.7	130
10	−2.5	15.2	11	−2.0	15.0	10
5	−1.3	14.2	4	−1.3	14.7	7
Controls	−0.5	13.6		−0.5	13.6	

BDF_1 mice were inoculated i.v. with 10^5 leukemic cells on day 0. Twenty-four hr after tumour inoculation, drugs were administered i.v. once a day for 4 days.
B.W.C. = body weight change on day 5.
Med.S.T. = median survival time of 8 treated mice and 16 untreated control mice.
I.L.S. = increase in life span of treated groups as compared to control group.

Table 3. Therapeutic effect of 2-FPTS and its Zn chelate (2-FPTS-Zn) administered i.p. on the life span of BDF_1 mice bearing L1210 ascitic leukemia

	2-FPTS			2-FPTS-Zn		
Doses mg/kg	B.W.C. (g)	Med.S.T. (days)	I.L.S. (%)	B.W.C. (g)	Med.S.T. (days)	I.L.S. (%)
12.5	−0.6	12	43	−2.2	14.8	76
6.25	−0.2	12.5	49	+0.6	11.2	33
3.12	+0.4	11.6	38	+1.0	10.2	21
Saline	+1.5	8.4		+1.5	8.4	

Male BDF_1 mice weighing 23–26 g were inoculated i.p. with 10^5 leukemic cells on day 0 and were treated with one injection a day for 9 days after tumour transplantation.
B.W.C. = body weight change on day 5.
Med.S.T. = median survival time of 8 treated animals and 32 controls.
I.L.S. = increase in life span of treated mice as compared to control group.

Table 4. Influence of the schedule and route of treatment on the therapeutic activity of 2-FPTS against L1210 leukemia

Treatment		Dose range mg/kg/day	Optimal dose mg/kg/day	Med.S.T. (days)	I.L.S. (%)	% survival after day 50	
Route	Schedule					Leukemic mice	Intact mice*
i.p.	once, day 1	25–100	50	9	0	0	100
i.p.	Q3h, day 1	3.75 × 8–30 × 8	7.5	14.5	61	0	100
i.p.	days 1, 5, 9	12.5–50	25	9	0	0	100
i.p.	Q3H, days 1, 5, 9	3 × 8–24 × 8	6	17	89	0	87.5
i.p.	days 1–9	3–50	12	13	44	0	100
i.p.	days 1, 9	12.5–75	50	9	0	0	75
i.p.	Q3H, days 1, 9	3 × 8–24 × 8	6	15	67	0	87.5
p.o.	once, day 1	25–200	100	9	0	0	100
p.o.	days 1–9	6.25–50	12.5	9	0	0	100
		Untreated		9		0	100

BDF_1 mice received 10^5 leukemic cells on day 0.
Med.S.T. = median survival time of 10 leukemic treated mice and 94 untreated controls.
I.L.S. = increase in life span of treated groups as compared to control groups.
*2 groups of 8 intact (non-leukemic) mice were treated as well as the leukemic mice in order to check the toxicity of the drug on intact mice and the mortalities due to manipulation.

Table 5. Influence of the schedule and route of treatment on the therapeutic activity of 2-FPTS-Zn against L1210 leukemia

Treatment		Dose range mg/kg/day	Optimal dose mg/kg/day	Med.S.T. (days)	I.L.S. (%)	% survival after day 50	
Route	Schedule					Leukemic mice	Intact mice*
i.p.	once, day 1	75–300	90	9.9	4	0	100
i.p.	Q3h, day 1	6.25 × 8–50 × 8	12.5	15.4	62	0	100
i.p.	days 1, 5, 9	50–200	50	11	16	0	100
i.p.	Q3h, days 1, 5, 9	3.12 × 8–18.75 × 8	6.25	23.9	151	40	100
i.p.	days 1–5	3–60	15	12.5	31	0	100
i.p.	days 1–9	3–50	14	13.7	44	0	87.5
i.p.	days 1, 9	75–200	75	12.5	31	0	75
i.p.	Q3h, days 1, 9	6.25 × 8–37.5 × 8	12.5	16.4	73	0	87.5
p.o.	once, day 1	200–300	200	9.4	0	0	100
p.o.	days 1–9	25–5	50	11.6	22	0	100
		Untreated		8.9		0	

BDF_1 mice received 10^5 leukemic cells on day 0.

Med.S.T. = median survival time of treated leukemic mice and 60 untreated controls.

I.L.S. = increase in life span of treated groups as compared to control group.

*Groups of 8 intact non-leukemic mice were treated as well as leukemic mice in order to check the toxicity of the drug and the mortalities due to manipulation.

Different chemical batches displayed a similar therapeutic activity when administered p.o. or i.p. to leukemic mice.

2. *Therapeutic effect of the 2-formylpyridine thiosemicarbazone* Zn *sulfate on melanoma B*16

The 2-FPTS-Zn sulphate was inactive against Melanotic melanoma B16 implanted subcutaneously (s.c.) in mice.

3. *Effect of 2-FPTS-*Zn *chelate on tumour growth, lung metastases and survival of mice bearing Lewis lung carcinoma i.m.*

The i.p. treatment with 12 mg/kg of 2-F.P.T.-Zn chelate for 15 days prolonged survival time of 49% over controls and 3 out of 8 mice survived after day 60 (Table 6). The other two doses did not influence the survival of treated mice. The 12, 6 and 3 mg/kg doses slightly inhibited tumour growth and strongly inhibited the formation of lung metastases (Table 7).

dependent, which property may be due to the rapid excretion of the drug. The slight therapeutic effect against the blood form of L1210 leukemia, when the drug was injected i.v., is due apparently to the schedule depending propriety of the drug. We could not administer i.v. more than 1 injection a day for only 4 days, which is not a good schedule, but it was practically difficult to choose a better one in this case.

The similar therapeutic effectiveness yielded when different chemical batches were used corroborates the fact that the synthesis is well standardized and reproducible.

Although our data do not provide any explanation for the enhanced therapeutic activity against P388 and L1210 leukemias of the chelate over the free drug, there is a possible interpretation that, however purely hypothetical, could be investigated:

(a) Zn is necessary for the nucleid acid synthesis [14–16]. An excess of Zn may also be

Table 6. *Effect of 2-FPTS-*Zn *chelate on the survival of mice bearing Lewis lung carcinoma i.m.*

Doses mg/mk	B.W.C. (g)	Med.S.T. (days)	I.L.S. (%)	% survivors after day 60
12	−3.9	46.3	49	37.5
6	−2.5	31.5	1	0
3	+0.1	32.4	3	0
Saline	+0.7	31.3		0

Male BDF_1 mice were inoculated i.m. with 2×10^6 tumour cells. Animals received one injection a day from day 1 to day 15 after tumour implantation.
B.W.C. = body weight change on day 5.
Med.S.T. = median survival time of 8 treated mice and 34 untreated controls.
I.L.S. = increase in life span of treated mice as compared to control group.

DISCUSSION

This study described the preparation of a Zn chelate with 2-formylpyridine thiosemicarbazone and its antineoplastic activity against experimental tumours.

Our data show that the Zn chelate of 2-formylpyridine thiosemicarbazone has a higher therapeutic activity than the free drug against P388 and L1210 leukemias. This increased therapeutic activity against L1210 leukemia of 2-formylpyridine Zn coordination compound (chelate) as compared to free 2-formylpyridine thiosemicarbazone was more apparent when the every 3 hr every 4 days schedule was used. We may assume from our data that the therapeutic activity against L1210 leukemia of FPTS-Zn is schedule

deleterious to cell proliferation. Zn salts prevent the initiation of Sarcoma 180 growth [17] and inhibit the growth of L1210 leukemia in mice [12]. Therefore, an additional therapeutic effect could be originated by incorporating Zn in molecules of 2-FPTS. A comparative study between the 2-formylpyridine thiosemicarbazone zinc sulphate containing 19.132% of Zn in the complex and the 2-formylpyridine thiosemicarbazone zinc chloride containing 23.14% of Zn in the complex showed that the chloride had a higher therapeutic activity than the sulphate against L1210 leukemia (unpublished data, Atassi *et al.*) and this corroborates our hypothesis.

(b) Studies on the molecular mechanism of action of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones suggest a model according

Table 7. Effect of 2-FPTS-Zn chelate on Lewis lung carcinoma and lung metastases

Doses mg/kg	Average body weight on day of sacrifice (g)	Average tumour weight (g)	Mice with tumour		Mice with metastases		Average weight of metastases per mouse (mg)
			No. of mice in the group		Mice with tumour		
12	20.90 ± 1.5	4.50 ± 1.2	5/5		3/5		1.25
6	23.23 ± 2.4	7.27 ± 1.8	7/7		7/7		13.22
3	25.31 ± 2.5	8.90 ± 1.5	7/7		7/7		52.53
Saline	25.47 ± 2.6	9.30 ± 1.3	9/9		9/9		116.0

Male BDF_1 mice were inoculated i.m. with 2×10^6 tumour cells on day 0. The drug was injected i.p. from days 1 to 15 after tumour implantation. Animals were sacrificed on day 24.

to which these agents bind, as tridentate ligands, either to an iron-charged ribonucleoside-diphosphate reductase or to a preformed iron chelate of α-(*N*)-heterocyclic carboxaldehyde which is the inhibitor of the enzyme and interacts with the target enzyme [18]. These properties may have negative side-effects such as hemolyses and excretion of iron. We may imagine that the previous chelation of the inhibitor will protect red blood cells and bind preferentially to an iron-charged ribonucleoside-diphosphate reductase. This also suggests to use the iron-chelate instead of the Zn-chelate, which is under investigation. Our primary results with the iron-chelate (Fe II) are very encouraging since this chelate shows a therapeutic activity against P388 leukemia (unpublished data, Atassi *et al.*) similar to that of the Zn-chelate but was inactive against L1210 leukemia.

The melanoma B16 was insensitive to the drug under the conditions of the experiment contrary to the Lewis lung carcinoma. However, in the case of the latter solid tumour, the inhibitory effect on the formation of lung metastases was much more apparent than that on the primary tumour, which suggests that the drug is more effective against free separated tumour cells than against mass of tumour.

The therapeutic effectiveness of the 2-FPTS-Zn chelate appears to depend on the nature of the tumour and to require intimate and rather long contact between the drug and the tumour cells, which may underline the enhanced activity against leukemia and migrating cells on their way from the primary tumour to the organ where they will form the metastases, when such contact is possible.

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