Cancer Chemother Pharmacol (1992) 29: 190–194

Cancer Chemotherapy and Pharmacology

© Springer-Verlag 1992

Antitumor drug cross-resistance in vivo in a murine P388 leukemia resistant to ethyl 5-amino-1,2-dihydro-2-methyl-3-phenylpyrido[3,4-*b*]pyrazin-7-ylcarbamate 2-hydroxyethanesulfonate hydrate (NSC 370147) 370147

William R. Waud[1], Steadman D. Harrison Jr.[1], Carroll G. Temple Jr[2], and Daniel P. Griswold Jr.[1]

[1] Chemotherapy and Toxicology Research and [2] Organic Chemistry Research, Southern Research Institute, Birmingham, AL 35255–5305, USA

Received 2 January 1991/Accepted 14 August 1991

Summary. Ethyl 5-amino-1,2-dihydro-2-methyl-3-phenylpyrido[3,4-*b*]pyrazin-7-ylcarbamate 2-hydroxyethanesulfonate hydrate (NSC 370147) is a potent mitotic inhibitor, which has provided the basis for a candidate for clinical trial. As observed with clinically useful drugs, the development of clinical resistance to NSC 370147 will probably be encountered. Information concerning resistance to NSC 370147 should aid in the design of strategies for the opitmal clinical use of the drug. A P388 leukemia resistant to NSC 370147 (P388/NSC 370147) was isolated and its in vivo cross-resistance profile was determined. The P388/NSC 370147 line was cross-resistant to vincristine but was not cross-resistant to doxorubicin, etoposide, cisplatin, melphalan, methotrexate, or 5-fluorouracil. This information plus other in vivo cross-resistance data [Waud et al. (1990) Cancer Res 50: 3239] suggests that NSC 370147 may be useful in non-cross-resistant combinations with doxorubicin, melphalan, cisplatin, or methotrexate. The lack of cross-resistance of P388/NSC 370147 to doxorubicin and etoposide shows that resistance to NSC 370147 does not involve multidrug resistance and suggests that the *mdr1* gene is not involved in resistance to NSC 370147.

Introduction

A series of 1,2-dihydropyrido[3,4-*b*]pyrazines was synthesized by Temple and coworkers as candidate antifolic acid compounds [6, 7]. The compounds were shown to have modest activity against P388 leukemia in vivo [6, 7]; however, they did not function as analogues of folic acid but instead inhibited mitosis and caused cultured cells to accumulate in metaphase [10]. Further studies showed that members of this series inhibited the polymerization of tubulin and competed with colchicine for binding to tubulin [1, 11].

Ethyl 5-amino-1,2-dihydro-2-methyl-3-phenylpyrido.[3,4-*b*]pyrazin-7-ylcarbamate 2-hydroxyethanesulfonate hydrate (NSC 370147), one of the more active compounds in the series, was selected for further evaluation. NSC 370147 was cytotoxic to a variety of mouse and human cell lines at nanomolar concentrations [8]. The compound exhibited good in vivo antitumor activity against several murine tumors (P388 and L1210 leukemia, colon 11/A and 36, mammary 16/C, and M5076 sarcoma). Activity was largely independent of route of administration but favored a prolonged treatment schedule. NSC 370147 was as active against murine leukemia sublines resistant to doxorubicin, amsacrine, vincristine, melphalan, cisplatin, methotrexate, and CI-920 (a topoisomerase II inhibitor) as against the corresponding parental lines. Because of the favorable antitumor activity of NSC 370147 in comparison to vincristine, its effectiveness against multidrug-resistant cells, and its retention of activity after oral administration, the compound has provided the basis for a candidate for clinical trial.

The development of resistance to clinically useful chemotherapeutic agents is a common occurrence. It seems reasonable to anticipate that resistance to NSC 370147 will be encountered clinically. Previous studies from our laboratory have shown that the in vivo cross-resistance profile of a drug-resistant P388 leukemia can yield useful insights into mechanisms of resistance, cross-resistance, and collateral sensitivity [9]. We report here the isolation of a P388 leukemia resistant to NSC 370147 and its in vivo cross-resistance profile to several clinically useful agents. The cross-resistance profile has permitted the identification of possible non-cross-resistant drug combinations with NSC 370147 and insights into resistance to NSC 370147.

Abbreviations: LD_{10}, dosage level producing 10% lethality; P388/NSC 370147, P388 leukemia resistant to NSC 370147; P388/O, P388 leukemia sensitive to NSC 370147; qd, daily; q3h ×3, every 3 h for a total of three injections

Offprint requests to: W. R. Waud, Southern Research Institute, P. O. Box 55305, Birmingham, AL 35255–5305, USA

Table 1. Isolation in vivo of a subline of P388 leukemia resistant to NSC 370 147[a]

No. of passage from parent sensitive line	Evaluation of resistance				
	Treatment of passage		Median ILS[b] (%)		
	Dosage (mg kg^{-1} dose^{-1})	Schedule	Passage no.	P388/O	P388/NSC 370 147
0– 4	0.25	Days 1–6	4	+73	+66
5– 8	0.25	Days 1–6	8	+80	+44
9–13	0.25	Days 1–6	14	+40	+41
14–23	0.10	q3h ×3, days 1–6[c]	23	+89	–5

[a] For the treated passage line, $CD2F_1$ mice were implanted i.p. with 10^7 cells on day 0 and treated i.p. as indicated. For evaluation of the degree of resistance of the subline, $CD2F_1$ mice were implanted i.p. with 10^6 P388/O or P388/NSC 370 147 cells on day 0 and treated i.p. at a dosage of 0.50 mg kg^{-1} dose^{-1} on days 1–9

[b] ILS, increase in life span

[c] q3h ×3, every 3 h for a total of three injections

Materials and methods

Drugs. Antitumor drugs were provided either by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program (DTP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI) (Bethesda, Md.) or the Organic Chemistry Department, Southern Research Institute.

Mice and P388 leukemia. BALB/c × DBA/2 F_1 ($CD2F_1$) mice were obtained from various commercial suppliers and housed in open-top, stainless-steel cages. Mice were allowed commercial mouse food and water ad libitum. P388/O was obtained from the DTP Tumor Repository, DCT, NCI (Frederick, Md.).

Isolation of P388/NSC 370 147. P388/NSC 370 147 was isolated as described in Table 1. $CD2F_1$ mice were implanted i.p. with 10^7 P388/O cells on day 0 and treated i.p. as indicated. The subline was passaged once a week. After the 23rd passage, the subline was not treated. The degree of resistance of the subline was evaluated with each passage. $CD2F_1$ mice were implanted i.p. with 10^6 P388/O or P388/NSC 370 147 cells on day 0 and treated i.p. at a dosage of 0.50 mg kg^{-1} dose^{-1} on days 1–9.

Cross-resistance studies. P388/NSC 370 147 was used between the 4th and 15th passages after cessation of drug treatment. For the cross-resistance studies, $CD2F_1$ mice were implanted i.p. with 10^6 cells of either P388/O or P388/NSC 370 147 on day 0. Drugs were administered i.p. according to the schedules listed in the tables. Each drug was evaluated at several dosage levels (ranging from toxic to nontoxic) with each dosage level administered to 10 mice. Tumor-bearing control mice (20/experiment) were untreated. Mice were observed for life span. In each experiment, tumored groups were treated with a range of dosages of NSC 370 147 to confirm the resistance of the P388/NSC 370 147. Moreover, P388/NSC 370 147 was compared directly in each experiment to P388/O, and the parallel groups of mice were treated identically with a single drug preparation. Experiments were repeated for confirmation.

Quantification of antitumor activity. Antitumor activity was assessed on the basis of the percentage median increase in life span and net $\log_{10}$ cell kill. Calculations of net $\log_{10}$ cell kill were made from the tumor doubling time, which was determined from an internal tumor titration consisting of implants from serial tenfold dilutions [5]. Long-term (48- to 60-day) survivors were excluded from calculations of increase in life span and tumor cell kill. To assess tumor cell kill at the end of treatment, the survival time difference between treated and control groups was adjusted to account for regrowth of tumor cell populations that may occur between individual treatments [4]. The net $\log_{10}$ cell kill was calculated as follows:

$$\text{Net } \log_{10} \text{ cell kill} = \frac{(T - C) - (\text{duration of treatment in days})}{3.32 \times t_d}$$

where (T – C) is the difference in the median time to death (days) between the treated (T) and the control (C) groups and t_d is the mean tumor doubling time (days) calculated from a log/linear least-squares fit of the implant sizes and the median times of death of the titration groups.

Cross-resistance. Cross-resistance was defined as decreased sensitivity (by >2 $\log_{10}$ units of cell kill) of P388/NSC 370 147 leukemia to a drug compared to that observed concurrently in P388/O leukemia. Similarly, marginal cross-resistance was defined as a decrease in sensitivity of approximately 2 $\log_{10}$ units.

Results

The isolation of P388/NSC 370 147 is summarized in Table 1. Treatment of P388/O with NSC 370 147 at a dosage of 0.25 mg kg^{-1} dose^{-1}, every day for a total of six injections (qd ×6), for 13 weekly passages failed to select a P388 subline resistant to NSC 370 147. However, further treatment with the drug at a similar total dosage but different schedule (0.10 mg kg^{-1} dose^{-1}: q3h ×3, qd ×6) selected a subline resistant to NSC 370 147 after only 5 additional treated passages. Treatment of the subline with the drug was continued for an additional 4 passages before evaluating fully the degree of resistance to NSC 370 147. The tumor doubling time of the subline (0.50 day) was similar to that for P388/O (0.44 day).

The degree of resistance of P388/NSC 370 147 to NSC 370 147 is summarized in Table 2. The subline was marginally resistant and markedly resistant to NSC 370 147 at an optimal ($\leq LD_{10}$) dosage on the day-1-only and on the day-1–9 schedules respectively. Subsequent experiments using the day-1-only schedule at an optimal dosage yielded a decrease in sensitivity of P388/NSC 370 147 in comparison to P388/O of 2.9 ± 0.5 $\log_{10}$ units (mean ± SD, $n = 3$; data not shown). The subline was also resistant to NSC 370 147 using a day-1–5 schedule at an optimal dosage (decrease in sensitivity of P388/NSC 370 147 in comparison to P388/O of 2.9 ± 0.5 $\log_{10}$ units, $n = 4$; data not shown). The subline was marginally cross-resistant to vincristine.

The stability of the resistance to NSC 370 147 of P388/NSC 370 147 was evaluated after 17 weekly in vivo passages without drug treatment. The subline was un-

Table 2. Resistance of P388/NSC 370 147[a]

Drug	Dosage i.p. (mg kg^{-1} dose^{-1})	Schedule	Therapeutic response					
			P388/O			P388/NSC 370 147		
			Median ILS[b] (%)	Approx. $\log_{10}$ change in tumor burden after last treatment[c]	60-day survivors	Median ILS[b] (%)	Approx. $\log_{10}$ change in tumor burden after last treatment[c]	60-day survivors
NSC 370 147	7.5	Day 1 only	+63	–4.1	0/10	+30	–2.1	0/10
	5.0		+52	–3.4	0/10	+21	–1.5	0/10
	0.75	Days 1–9	+121	–2.4	0/10	–14	+2.3	0/10
	0.50		+89	–0.3	0/10	–5	+2.1	0/10
	0.33		+63	+1.3	0/10	–5	+2.1	0/10
Vincristine	3.0	Days 1, 5, 9	+200	–6.7	0/10	+139	–4.8	0/10
	2.5		+163	–5.1	0/10	+117	–3.3	0/10
	2.0		+147	–4.1	0/10	+108	–2.7	0/10

[a] $CD2F_1$ mice were implanted i.p. with 10^6 cells of P388/O or P388/NSC 370 147 leukemia on day 0. The tumor doubling times for P388/O and P388/NSC 370 147 leukemia were 0.44 and 0.50 day respectively. The mean survival times of control mice bearing P388/O and P388/NSC 370 147 implants were 9.5 and 11.5 days respectively

[b] ILS, increase in life span

[c] $\log_{10}$ change in viable tumor stem cell population at the end of therapy as compared to that at the start of therapy, based on the median day of death among the animals that died

Table 3. Cross-resistance of P388/NSC 370 147 to clinically useful drugs[a]

Drug	Optimal i.p. dosage[b] ($\leq LD_{10}$) (mg kg^{-1} dose^{-1})	Therapeutic response						Cross-resistance
		P388/O			P388/NSC 370 147			
		Median ILS[c] (%)	Approx. $\log_{10}$ change in tumor burden after last treatment[d]	48-day survivors	Median ILS[c] (%)	Approx. $\log_{10}$ change in tumor burden after last treatment[d]	48-day survivors	
Doxorubicin	15.0	+90	–7.0	3/10	+80	–6.9	4/10	No
	15.0	+115	–6.7	2/10	+130	–6.6	2/10	
Vincristine	2.0	+93	–6.1	1/10	+48	+0.5	0/10	Yes
	2.0	+104	–2.5	0/10	+52	+1.3	0/10	
	1.5	+118	–2.7	0/10	+40	+1.3	0/10	
Etoposide	40.0	+93	–6.1	9/10	+106	–6.9	9/10	No
	40.0	+260	–6.7	7/10	+200	–6.6	5/10	
Cisplatin	8.0	+103	–7.0	0/10	+135	–6.9	2/10	No
	8.0	+250	–6.7	0/10	+220	–6.6	4/10	
Melphalan	20.0	+163	–6.5	6/10	+73	–6.6	2/10	No
	20.0	+131	–6.7	6/10	+73	–6.0	4/10	
Methotrexate	2.5	+100	–1.6	0/10				No
	3.0				+63	–1.0	0/10	
	4.0	+90	–1.4	0/10	+108	–3.2	0/10	
5-Fluorouracil	30.0	+81	–2.7	0/10	+50	–2.3	0/10	No
	35.0	+59	–1.8	0/10	+60	–2.1	0/10	

[a] $CD2F_1$ mice were implanted i.p. with 10^6 cells of P388/O or P388/NSC 370 147 leukemia on day 0. The tumor doubling times for P388/O and P388/NSC 370 147 leukemia were 0.43 ± 0.10 day and 0.44 ± 0.08 day (mean $\pm$ SD, $n = 4$) respectively. The median survival times of control mice bearing P388/O and P388/NSC 370 147 implants were 11.8 ± 2.2 days and 13.0 ± 2.7 days respectively ($n = 4$)

[b] Doxorubicin and melphalan were administered on day 1; vincristine, etoposide, and cisplatin were administered on days 1, 5, and 9; methotrexate was administered on days 1–9; 5-fluorouracil was administered on days 1–5

[c] ILS, increase in life span

[d] $\log_{10}$ change in viable tumor stem cell population at the end of therapy as compared to that at the start of therapy, based on the median day of death among the animals that died

changed in its resistance to NSC 370 147 and its tumor doubling time; however, the subline was more resistant to vincristine than before the cessation of drug treatment (decrease in sensitivity of P388/NSC 370 147 to vincristine in comparison to P388/O of 3.1 $\log_{10}$ units at a dosage of 3.0 mg kg^{-1} dose^{-1} on days 1, 5, and 9; data not shown).

The in vivo cross-resistance profile of P388/NSC 370147 (passaged in the absence of drug) to seven clinically useful drugs is shown in Table 3. The P388/NSC 370147 line was cross-resistant to vincristine but was not cross-resistant to doxorubicin, etoposide, cisplatin, melphalan, methotrexate, or 5-fluorouracil.

Discussion

Because of the favorable antitumor activity of NSC 370147 in comparison to vincristine, its effectiveness against multidrug-resistant cells, and its retention of activity after oral administration, the compound has provided the basis for a candidate for clinical trial. As observed with clinically useful drugs, the development of clinical resistance to NSC 370147 will probably be encountered. Information concerning resistance to NSC 370147 should aid in the design of strategies for the optimal clinical use of the drug.

Studies from our laboratory have shown that the antitumor activity of NSC 370147 in preclinical models favored a prolonged treatment schedule (e.g., q3h $\times 8$ on days 1, 5, 9) [8]. Accordingly, a P388 subline resistant to NSC 370147 was obtained only after using a prolonged treatment schedule (e.g., q3h $\times 3$ on days 1–6). Interestingly, the drug-resistant subline was selected with a treatment that was similar in total dosage and percentage increase in life span (for P388/O) to a treatment (0.25 mg kg^{-1} $dose^{-1}$, days 1–6) that failed to select a drug-resistant subline. In reviewing the isolation protocols for the 20 drug-resistant P388 leukemias that we have at Southern Research Institute, only P388/NSC 370147 required multiple injections on each day of treatment. P388 leukemia resistant to another mitotic inhibitor, vincristine, was obtained using multiple courses of q4d $\times 3$ treatment. P388 leukemias resistant to agents that require a prolonged treatment schedule for optimal antitumor activity (e.g., methotrexate or 1-β-D-arabinofuranosylcytosine) were obtained using multiple courses of qd $\times 9$ treatment (unpublished data). The resistance of the subline to NSC 370147 was stable for at least 17 weekly passages; however, the degree of cross-resistance of the subline to vincristine increased during passage without treatment with NSC 370147.

We have reported previously that treatment of doxorubicin-, vincristine-, melphalan-, cisplatin-, and methotrexate-resistant P388 leukemias with NSC 370147 resulted in cell kills comparable to that obtained by treating the sensitive P388/O line [8]. This information plus the in vivo cross-resistance profile reported here suggests that NSC 370147 may be useful in non-cross-resistant combinations with doxorubicin, melphalan, cisplatin, or methotrexate. Previous studies from our laboratory, which have shown that NSC 370147 is synergistic in combination with vincristine against L1210 (in vitro) and P388 leukemias (in vivo) [1], suggest that the combination of NSC 370147 and vincristine may also be useful clinically. However, the cross-resistance of P388/NSC 370147 to vincristine may lessen the usefulness of this combination. As always, none of these approaches may be applied clinically without caution and concern for the recognized gap between preclinical prediction and clinical validation.

The lack of cross-resistance of P388/NSC 370147 to doxorubicin and etoposide shows that resistance to NSC 370147 does not involve multidrug resistance and suggests that the *mdr1* gene is not involved in resistance to NSC 370147. Preliminary studies have shown that P388/NSC 370147 cells do not have an amplified *mdr1* gene or an increased level of P glycoprotein (unpublished data). Therefore, the observed cross-resistance of P388/NSC 370147 to vincristine does not require either multidrug resistance or overexpression of the *mdr1* gene. Whereas this is not the typical situation with resistance to vincristine, there are reports of similar findings. A vincristine-resistant murine L5178Y leukemia, selected in vitro by exposure to X-irradiation, exhibited cross-resistance to actinomycin D and vinblastine but not to doxorubicin [2]. A vincristine-resistant human rhabdomyosarcoma xenograft, selected in vivo by exposure to vincristine, showed no increase in *mdr 1* mRNA in comparison to the parental xenograft [3].

In summary, a P388 leukemia resistant to NSC 370147 has been isolated and its in vivo cross-resistance profile has been determined. The cross-resistance profile has permitted the identification of possible non-cross-resistant drug combinations with NSC 370147 and insights into in vivo resistance to NSC 370147. Further studies will be required to confirm that NSC 370147 resistance is independent of *mdr1* gene expression and to elucidate the mechanism(s) by which P388 cells are resistant to this drug in vivo.

Acknowledgements. This work was supported by grant PO1-CA34200 awarded by the NCI, National Institutes of Health, Department of Health and Human Services. The authors gratefully acknowledge the technical assistance of the staff of the Drug Formulation and In Vivo Evaluation Section. Jacquelyn Tubbs and Gail Jones assisted with data management. Sheree Robinette prepared the manuscript.

References

1. Bowdon BJ, Waud WR, Wheeler GP, Hain R, Dansby L, Temple C Jr (1987) Comparison of 1,2-dihydropyrido[3,4-*b*]pyrazines (1-deaza-7,8-dihydropteridines) with several other inhibitors of mitosis. Cancer Res 47: 1621
2. Hill BT, Whelan RDH, Bellamy AS (1984) Identification of differential drug responses and mechanism of resistance in vincristine-resistant cell lines developed either by exposure to the drug or to fractionated radiation. Cancer Treat Rev 11 [Suppl A]: 73
3. Horton JK, Houghton PJ, Houghton JA (1987) Reciprocal cross-resistance in human rhabdomyosarcomas selected in vivo for primary resistance to vincristine and L-phenylalanine mustard. Cancer Res 47: 6288
4. Lloyd HH (1977) Application of tumor models toward the design of treatment schedules for cancer chemotherapy: In Drewinko B, Humphrey RM (eds) Growth kinetics and biochemical regulation of normal and malignant cells. Williams & Wilkins, Baltimore, p 455
5. Schabel FM Jr, Griswold DP Jr, Laster WR Jr, Corbett TH, Lloyd HH (1977) Quantitative evaluation of anticancer agent activity in experimental animals. Pharmacol Ther [A] 1: 411
6. Temple C Jr, Wheeler GP, Elliott RD, Rose JD, Kussner CL, Comber RN, Montgomery JA (1982) New anticancer agents: synthesis of 1,2-dihydropyrido[3,4-*b*]pyrazines (1-deaza-7,8-dihydropteridines). J Med Chem 25: 1045

7. Temple C Jr, Wheeler GP, Elliott RD, Rose JD, Comber RN, Montgomery JA (1983) 1,2-Dihydropyrido[3,4-*b*]pyrazines: structure-activity relationships. J Med Chem 26: 91
8. Waud WR, Leopold WR, Elliott WL, Dykes DJ, Laster WR Jr, Temple CG Jr, Harrison SD Jr, Griswold DP Jr (1990) Antitumor activity of ethyl 5-amino-1,2-dihydro-2-methyl-3-phenylpyrido[3,4-*b*]pyrazin-7-ylcarbamate, 2-hydroxyethanesulfonate, hydrate (NSC 370 147) against selected tumor systems in culture and in mice. Cancer Res 50: 3239
9. Waud WR, Harrison SD Jr, Gilbert KS, Laster WR Jr, Griswold DP Jr (1991) Antitumor drug cross-resistance in vivo in a cisplatin-resistant murine P388 leukemia. Cancer Chemother Pharmacol 27: 456
10. Wheeler GP, Bowdon BJ, Werline JA, Adamson DJ, Temple CG Jr (1982) Inhibition of mitosis and anticancer activity against experimental neoplasms by ethyl 5-amino-1,2-dihydro-3-[(*N*-methylanilino)methyl]pyrido[3,4-*b*]pyrazin-7-ylcarbamate (NSC 181928). Cancer Res 42: 791
11. Wheeler GP, Bowdon BJ, Temple C Jr, Adamson DJ, Webster J (1983) Biological effects and structure-activity relationships of 1,2-dihydropyrido[3,4-*b*]pyrazines. Cancer Res 43: 3567