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N-Methylformamide (NSC 3051): a Potential Candidate for Combination Chemotherapy*

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Abstract—*N-Methylformamide (NMF) was found to be non-toxic to the bone marrow as reflected in the absence of leukopenia in mice, even when the marrow had been compromised by prior administration of cyclophosphamide. Thus recovery from the leukopenic nadir after* 160 mg/kg *of cyclophosphamide was unaffected by* 200 mg/kg × 10 *of NMF. This combination, given to animals bearing the M5076 sarcoma, proved to have an additive antitumour effect as measured by tumour growth delay and was superior to the antitumour effect of two doses of cyclophosphamide, a regime which prolonged the leukopenia. Furthermore, the hepatotoxicity of NMF was not augmented by the addition of cyclophosphamide. When hepatotoxicity was induced in BALB/c mice bearing the NMF-resistant ADJ/PC6A plasmacytoma, cyclophosphamide fully maintained its antitumour effect. The results show NMF to be a highly specific antiproliferative agent with potential for use in the therapy of patients with a compromised bone marrow and/or in combination chemotherapy.*

INTRODUCTION

DRUG combination chemotherapy is being employed increasingly in the treatment of cancer. The objective of combination chemotherapy is to derive a therapeutic advantage over single-agent therapy alone. One means by which this end may be achieved is to combine drugs of non-overlapping and independent toxicities.

For many of the widely used antitumour agents, for example cyclophosphamide [1], nitrogen mustard [2] and methotrexate [3], myelosuppression often proves to be dose-limiting. *N*-Methylformamide (NMF; Fig. 1), which is currently being used in phase 1 and phase 2 trials, both in Europe and the U.S.A., has not exhibited this toxicity in man nor in any other species investigated to date [4–7]. Early clinical indications suggest that reversible hepatotoxicity and gastrointestinal toxicity probably represent its major side effects in man [5, 7–9]. In rodents and dogs this reversible hepatotoxicity also represents the major toxicity of NMF [6, 7, 10]. This suggested two circumstances in which NMF might be used advantageously. Firstly, in combination with chemotherapeutic agents of non-overlapping toxicity, and secondly, in circumstances where the bone marrow had previously been compromised and thus where no myelosuppressive drug might be used safely. To investigate these two situations we have studied the effects of NMF on the recovery of bone marrow compromised by prior administration of cyclophosphamide (CY). The myelosuppression induced by CY has been studied previously in mice [11] and the leukopenia shown to correlate precisely with bone marrow destruction. Thus in this study we have used leukocyte counts as an indicator of myelosuppression.

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Abbreviations: CY, cyclophosphamide; NMF, *N*-methylformamide; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulphoxide; SDH, sorbitol dehydrogenase; ALT, L-alanine aminotransferase; AST, L-aspartate aminotransferase.

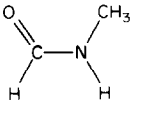

Fig. 1. Structure of N-methylformamide.

The reversible hepatotoxicity of NMF is an important factor in any study of the efficacy of drug combinations if one or more of the drugs requires hepatic metabolism for activation, as is the case with CY [12]. Accordingly we have studied the efficacy of CY against a murine plasmacytoma sensitive to the alkylating agent but resistant to NMF under conditions of NMF-induced hepatotoxicity.

MATERIALS AND METHODS

Drugs

NMF was obtained from Aldrich Chemicals, Gillingham, U.K. and purified further by redistillation. CY was obtained from Sigma Ltd, Poole, U.K. Drugs were administered to mice dissolved in the following: NMF in physiological saline and CY in DMSO:arachis oil (1:9).

Animals

Female BALB/c and BDF_1 (DBA/2 × C57/BL hybrid) mice (18–23 g) were purchased from Bantin and Kingman Ltd, Hull, U.K. They were fed on Heygates modified 41B breeding diet and allowed tap water *ad libitum*.

Antitumour assays: M5076 sarcoma

A suspension of 10^6 M5076 sarcoma cells from a routine passage (maintained as a solid s.c. tumour in mice) was injected i.m. into the left hind legs of groups of 10 female BDF_1 mice. Drugs were administered i.p. according to the schedules described in Table 1. Mean tumour volumes were determined in the following manner. Tumour diameters were first measured by calipers and the volumes calculated using the following formula:

$$\text{volume} = \frac{l \times w^2}{2},$$

where l represents the longest tumour diameter and w the diameter perpendicular to this axis [13]. The volume of the leg without tumour is 0.1–0.2 cm^3 when measured in this manner; thus tumours smaller than this value are referred to as non-measurable. For the combination experiments mice with tumours of 0.5–1.0 cm^3 were randomised into groups 12 days after implantation of the tumour. All tumours were palpable and measurable on this day (day 12); thus all groups were equivalent at the initiation of drug treatment.

ADJ/PC6A plasmacytoma assay

A suspension of 10^6 tumour cells (maintained in BALB/c mice in a manner identical to the M5076 sarcoma) was injected i.m. into the left hind legs of groups of five BALB/c mice. Drugs were administered according to the described schedules and tumour volumes measured as above.

Lethal dose values

Drugs were administered to groups of 5 or 10 mice at dose levels ranging from non-lethal to complete mortality. Mortality was assessed on day 30 post-final injection. Lethal dose values were calculated according to the method of Litchfield and Wilcoxon [14].

Peripheral blood cell counts

While BDF_1 mice bearing the M5076 sarcoma were under halothane anaesthesia blood samples were collected from the tip of the tail into blood cell pipettes. Blood was diluted in a 1% acetic acid/saline solution, stained with gentian violet and white blood cells counted using a Weber B.S.A. improved Neubauer haemocytometer. Platelets were counted in the same manner in a sodium citrate solution (3 g/100 ml) and were stained with methylene blue (1% in saline). The P values were obtained using Student's t test.

Determination of plasma enzyme activities

While non-tumour-bearing mice were under halothane anaesthesia blood samples (1 ml) were collected into heparinised syringes by exsanguination from the abdominal aorta at the iliac bifurcation. Immediately after this procedure the animals were killed. Plasma was obtained by centrifugation for 1 min using a Beckman microfuge. Sorbitol dehydrogenase (SDH) activity in the plasma was assayed by a method described by Rose and Henderson [15] while L-alanine aminotransferase (ALT) and L-aspartate aminotransferase (AST) activities were assayed according to methods described by Kachmar and Moss [16]. All enzyme activities were assayed at 37°C. Mice without tumours were used in these studies since destruction of the tumour by the drug might have influenced plasma enzyme activities. The P values were obtained by the Mann–Whitney U test.

RESULTS

Drug combination schedules

BDF_1 mice bearing the M5076 sarcoma were treated with NMF alone, CY alone or with combinations of the two drugs according to the schedules described in Table 1. Treatment was delayed until day 12 post-tumour implant, since the tumours are palpable and measurable on this day. NMF injections were initiated 3 days after CY treatment as the marrow cells and colony-forming units are reported to be most sensitive at this stage [11]. NMF exerts optimal antitumour activity

Table 1. Dosage schedules used in the combination experiments

Schedule	Treatment	NMF dose (mg/kg/day)	CY dose (mg/kg/day)	No. dead/total	White blood cell count (± S.E.)/mm³ (day 16)	Mean tumour volume (cm³ ± S.E.) Day 12	Mean tumour volume (cm³ ± S.E.) Day 40	Tumour volume delay*	LD_{10} (mg/kg/day)†
A	CY day 12	—	320	1/10	0.4 (±0.1) × 10^3	0.7 ± 0.1	3.4 ± 0.2	27	370
B	CY day 12	—	160	0/10	1.5 (±0.3) × 10^3	0.9 ± 0.1	5.8 ± 0.3	24	370
C	NMF days 15–24	200	—	0/10	7.5 (±0.5) × 10^3	0.8 ± 0.1	6.0 ± 0.4	22	480
D	CY day 12/NMF days 15–24	200	320	0/10	0.4 (±0.1) × 10^3	0.8 ± 0.1	NM‡	38	—
E	CY day 12/NMF days 15–24	200	160	0/10	1.2 (±0.3) × 10^3	0.9 ± 0.1	0.4 ± 0.1	35	—
F	CY days 12 and 15	—	160	0/10	0.8 (±0.1) × 10^3	0.9 ± 0.1	2.1 ± 0.4	30	—
G	untreated control	—	—	0/10	8.3 (±0.3) × 10^3	0.8 ± 0.1	—	8	—

*Time (days) for mean tumour volume to reach 4 × volume on day 12.

†Value for the schedule described.

‡NM = non-measurable, as defined in Materials and Methods.

against the M5076 sarcoma when administered by chronic schedules [4] and thus this type of regime was used in the combination experiments.

White blood cell counts

White blood cell counts for the various treatments are illustrated in Fig. 2. The nadir proved to be about 4 days after CY treatment, and white blood cell counts on this day are given in Table 1. NMF alone (schedule C, Table 1) produced no change from control values whereas CY at either 320 or 160 mg/kg (schedules A and B) produced a precipitous fall. The combination of CY and NMF (D and E) elicited identical recovery of the white cell count to that obtained when CY alone had been administered. No significant difference was found between schedules A and D or between B and E ($P > 0.01$). Repeated treatment with CY (schedule F) extended the leukopenia compared to a single injection.

Tumour volumes

The delays in the growth of mean tumour volumes after treatment are tabulated in Table 1 and several are illustrated in Fig. 3. Values in Table 1 are depicted as delay times to achieve 4 × initial tumour volume (day 12 value). The combination treatments (D and E) proved superior to the single agents alone or to repeated treatment with CY.

Animal weights

Mean weights of the animals bearing M5076 tumours are given in Table 2. These weights include the weight of the tumour (shown in brackets) which was calculated from the tumour

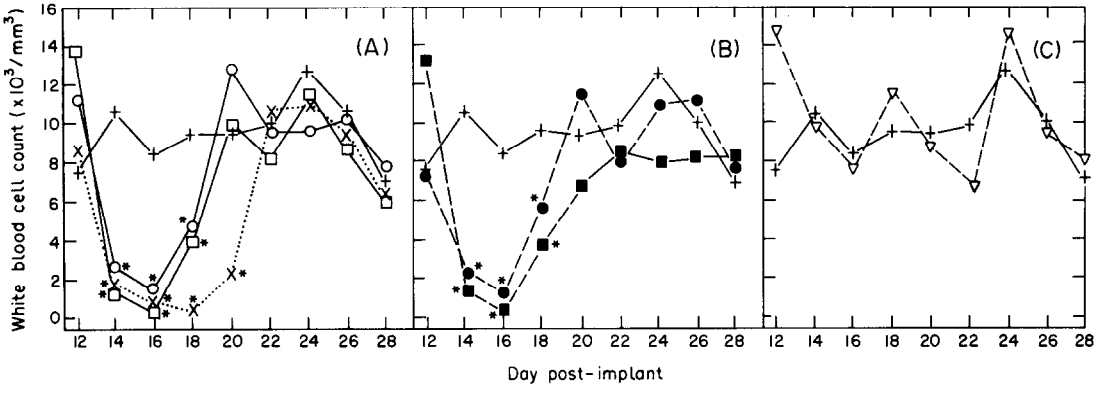

Fig. 2. Peripheral white blood cell counts of BDF_1 mice treated with the CY/NMF combination. Drug treatments were: in A: □——□, *CY* 320 mg/kg (*day* 12); ○——○, *CY* 160 mg/kg (*day* 12); ×·····×, *CY* 160 mg/kg/day (*days* 12 *and* 15); +——+, *untreated control; in B:* ■--■ *CY* 320 mg/kg (*day* 12) + *NMF* 200 mg/kg/day (*days* 15-24), ●--● *CY* 160 mg/kg (*day* 12) + *NMF* 200 mg/kg/day (*days* 15-24), +——+ *untreated control; in C:* ▽——▽ *NMF* 200 mg/kg/day (*days* 15-24), +——+, *untreated control.* **Significantly different from control values (Student's t test) at* $P < 0.01$.

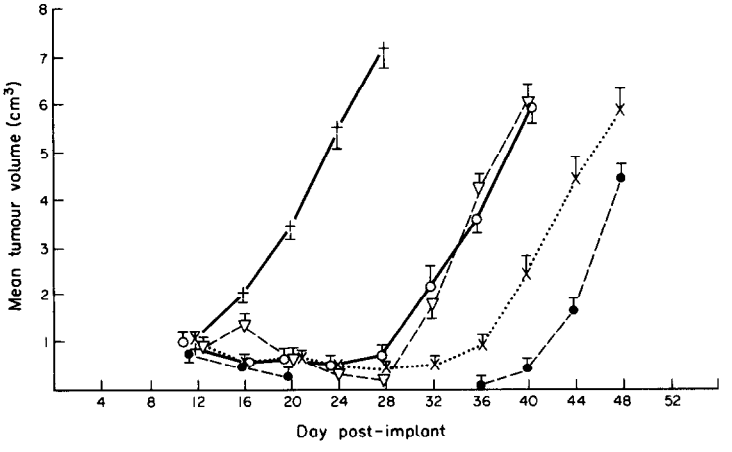

Fig. 3. Mean tumour volumes of BDF_1 mice bearing the M5076 sarcoma treated with the CY/NMF combination. Drug treatments were: ●--● *CY* 160 mg/kg (*day* 12) + *NMF* 200 mg/kg (*days* 15-24), ○——○ *CY* 160 mg/kg (*day* 12), ×·····× *CY* 160 mg/kg/day (*days* 12 *and* 15), ▽——▽ *NMF* 200 mg/kg/day (*days* 15-24), +——+ *untreated control. Each point represents the mean value* ± 1 *S.E.*

volume (assuming a density of 1). If allowance is made for the contribution of the tumour weight to the animal weight then the greatest weight loss induced by treatment was 1.9 g for CY alone (320 mg/kg schedule A). The combination schedules (D and E) were no more toxic as indicated by weight loss than was CY alone (A and B). The additive antitumour effects of NMF and CY thus cannot be attributed to an inhibition of tumour growth because of an increased cachexia of the animals given the drug combination.

Peripheral platelet counts

Platelet counts were monitored on alternate days in mice after the treatment schedules shown in Table 1. No significant deviations from control values were found in any of the treated mice ($P > 0.05$). Mean control (±S.E.) values ranged from 5.9 (±0.4) × 10^5/mm^3 to 7.2 (±0.7) × 10^5/mm^3.

SDH, ALT and AST activities

Plasma levels of three liver function enzyme markers were monitored after administration of the combinations to BDF_1 mice without tumours (Table 3). Increases in the activities of these enzymes in the plasma have previously been shown to correlate with histopathological changes in the liver [17]. The same schedules as described in Table 1 were used, with day 0 being equivalent to day 12. Control values were obtained for all of the above treatments, implying no significant levels of hepatotoxicity under these conditions. NMF at a dose of 400 mg/kg/day over 10 days was, however, hepatotoxic in BDF_1 mice (Table 3).

CY antitumour activity under conditions of NMF-induced hepatotoxicity

The dose levels at which NMF exerts hepatotoxicity in BALB/c mice were first established. At doses of 150 mg/kg or more of NMF hepatotoxicity was induced in non-tumour-bearing BALB/c mice, as indicated by increases of SDH, ALT and AST activities 24 hr after injection of drug (Fig. 4). This strain of mouse has previously been reported to be highly susceptible to the hepatotoxic effects of NMF [4, 17].

The ADJ/PC6A plasmacytoma is a transplantable murine tumour of BALB/c mice, sensitive to CY yet resistant to NMF. An experiment was conducted in which 250 mg/kg NMF was administered to a group of mice bearing the ADJ/PC6A tumour (implanted 13 days previously). This dose produces no antitumour effect (Fig. 5) yet induces hepatotoxicity (Fig. 4). Saline was given to a control group of mice bearing the tumour. CY was administered 24 hr later to both groups at doses varying between curative (>2.5 mg/kg) and inactive (<0.625 mg/

Table 2. Mean weights (g) of BDF_1 mice bearing the M5076 sarcoma used in the combination experiment

Schedule*	Day 12	Day 16	Day 20	Day 24
A	22.7 (0.7)†	20.5 (0.4)	21.4 (0.5)	21.4 (0.3)
B	22.2 (0.9)	20.4 (0.6)	22.3 (0.7)	20.1 (0.5)
C	22.6 (0.8)	23.0 (1.4)	22.7 (0.7)	22.0 (0.3)
D	23.0 (0.8)	21.5 (0.3)	21.3 (0.2)	21.9 (0)
E	22.9 (0.9)	21.9 (0.6)	22.8 (0.3)	23.0 (0)
F	22.8 (0.9)	21.7 (0.6)	21.6 (0.4)	23.2 (0.4)
G	22.0 (0.8)	23.6 (2.1)	24.9 (3.5)	27.0 (5.5)

*The schedules used are as described in Table 1.
†Approximate weight of tumour is given in parentheses.

Table 3. Plasma activities of SDH, ALT and AST in BDF_1 mice treated with the CY/NMF combination

Dose*		Plasma enzyme activity								
CY	NMF	SDH levels			ALT levels			AST levels		
(mg/kg/day)	(mg/kg/day)	Day 3†	Day 8	Day 13	Day 3	Day 8	Day 13	Day 3	Day 8	Day 13
320	200	0.9‡	1.1	0.8	0.9	0.9	0.7	0.6	0.7	1.3
160	200	0.9	1.0	1.5	1.2	0.8	0.7	1.0	0.7	1.0
320	—	0.9	1.1	—	0.9	0.8	—	0.6	1.3	—
160	—	0.9	0.9	—	1.2	1.2	—	1.0	0.8	—
—	200	—	1.4	1.3	—	1.2	1.0	—	0.9	1.2
—	400	—	17.9§	19.2§	—	15.1§	15.8§	—	3.5§	2.8§
—	—	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

*CY was administered i.p. on day 0; NMF administered i.p. on days 3–13.
†Values relative to control (= 1.0).
‡Plasma taken on the stated day was taken 24 hr after the previous injection.
§Significantly different from controls (Mann–Whitney U test) at $P < 0.05$).

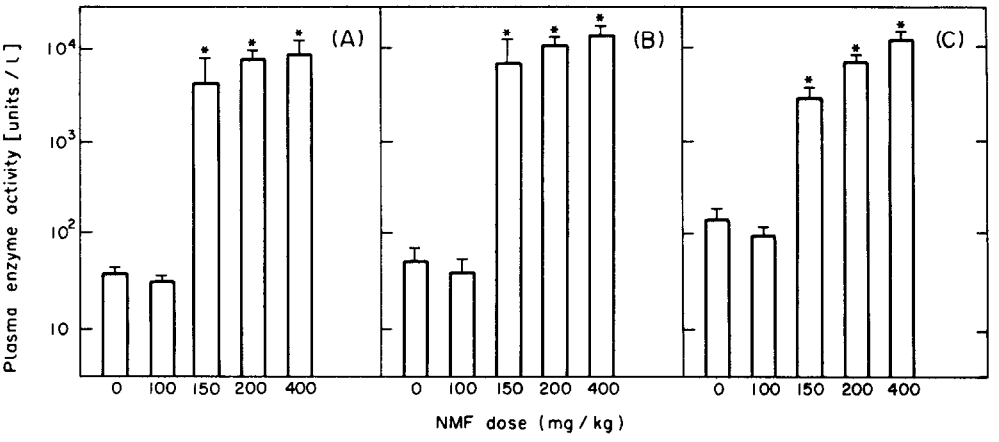

Fig. 4. Plasma activities of various enzymes in BALB/c mice 24 hr *after treatment with varying doses of NMF* [4]. *A, sorbitol dehydrogenase; B,* L-*alanine aminotransferase; C,* L-*aspartate aminotransferase.* **Significantly different from control values (Mann-Whitney* U *test) at* $P < 0.05$.

kg). Ten days later tumour volumes were measured and the tumour volumes of each group, namely those which had received CY + NMF and those which had received CY + saline, were compared with tumour-bearing mice which had received no drug (Fig. 5).

No significant difference ($P > 0.05$) was found between the group which had received CY + NMF and that which had received CY + saline. Thus under conditions of NMF-induced hepatotoxicity the antitumour effect of CY was unchanged.

DISCUSSION

NMF appears to be rare amongst the anti-neoplastic agents active against a range of murine tumours in that it has no effect on the bone marrow of mice, dogs or men [4-7]. If phase 2 trials show NMF to be active against human tumours, which it demonstrably is against human xenograft lines in mice [18], then this attribute will be of profound importance, particularly in the design of drug combination schedules.

The results presented here show that in BDF_1 mice which are bone marrow-compromised by the prior administration of 160 mg/kg of CY, NMF at a chronic dosage schedule does not exacerbate the profound leukopenia but instead allows bone marrow recovery equivalent to that of untreated animals (Fig. 2). In addition, the inhibition of the growth of the M5076 sarcoma by the combination of CY and NMF was greater than that for each drug alone under these conditions (Fig. 3). In comparison, a second treatment with CY to mice bearing the M5076 sarcoma not only extended the leukopenia (Fig. 2) but had less antitumour effect than the combination of NMF and CY (Fig. 3). When 320 mg/kg of CY was administered to BDF_1 mice bearing the sarcoma one mouse out of ten was killed. When 320 mg/kg CY was administered together with NMF (200 mg/kg × 10) no deaths occurred, suggesting that the combination is certainly no more toxic than CY alone. These results suggest that a therapeutic advantage might be obtained by use of a combination of the two agents.

One of the dose-limiting toxicities of NMF is hepatotoxicity, which is reversible [4, 5, 7]. The hepatotoxicity of the CY + NMF combination was examined in order to determine whether it rose above that of NMF alone. Plasma activities of the enzymes SDH, ALT and AST were determined in mice given the combination and were found to be the same as the values for NMF alone (Table 3). These results suggest that the combination of the

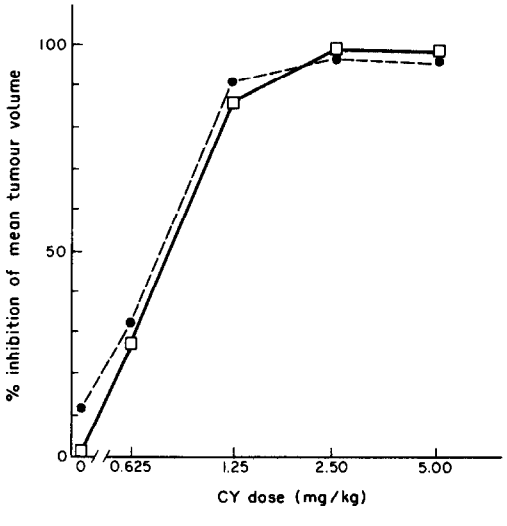

Fig. 5. Response of the ADJ/PC6A plasmacytoma to CY under conditions of NMF-induced hepatotoxicity. BALB/c mice were injected i.m. with 10^6 *tumour cells on day* 0. *On day* 13 *post-implant either NMF* (250 mg/kg) ●--● *or saline* □——□ *was administered to these mice. CY was injected* 24 hr *later (day* 14) *at the doses indicated and tumour volume inhibitions were measured on day* 24. *Tumour volume inhibitions of treated groups as compared with untreated control tumours are plotted for each dose of CY used.*

two drugs did not exacerbate their separate toxicities.

The toxicity of NMF to the liver raised the question as to whether hepatic damage could interfere with the metabolic activation of CY and consequently with its antitumour action. Drugs with the potential for hepatotoxicity such as methotrexate [19] and 6-mercaptopurine [20] have, however, previously been used in combination with CY. We chose to measure the effects of NMF on the antitumour efficacy of CY *in vivo* by studying the ADJ/PC6A plasmacytoma, a tumour highly sensitive to CY but totally resistant to NMF (Fig. 5). Hepatotoxicity in host BALB/c mice was induced by NMF, as measured by elevation of enzyme activities, then CY was administered. Figure 5 demonstrates that even when plasma enzyme activities were elevated, the therapeutic efficacy of CY was undiminished. As the largest dose of CY used was only about 1% of the LD_{10} (see Table 1) we cannot exclude the possibility that the metabolism of large doses, as commonly used in clinical practice, is affected by the co-administration of NMF.

The studies reported here suggest that if NMF is found to be active in phase 2 trials the drug may be an attractive candidate for therapy of those patients with a compromised bone marrow function and/or in combination therapy. Its complete lack of bone marrow toxicity and the recovery of a compromised bone marrow in the presence of a potent antitumour effect (Figs 2 and 3) raise important questions as to the mechanism of this remarkable specificity of action. We are continuing our efforts to attempt to answer this in the hope that we may synthesize analogues without hepatotoxicity and which thus have the potential for even greater selectivity.

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