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THE FORMATION AND METABOLISM OF *N*-HYDROXYMETHYL COMPOUNDS—IV

CYTOTOXICITY AND ANTITUMOUR ACTIVITY OF *N*-HYDROXYMETHYLFORMAMIDE, A PUTATIVE METABOLITE OF *N*-METHYLFORMAMIDE (NSC 3051)

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Abstract—Experiments were conducted to test the hypothesis that *N*-hydroxymethylformamide (HMF) is the active metabolite of the antitumour agent *N*-methylformamide (NMF). In an *in vitro* bioassay against the TLX5 lymphoma HMF was more cytotoxic than NMF; this cytotoxicity was abolished by preincubating the TLX5 cells with semicarbazide, a formaldehyde trapping agent. Similarly, the inhibition of incorporation of radiolabelled thymidine, uridine, formate and leucine into TLX5 cells elicited by HMF was eliminated by preincubation of the cells with semicarbazide. HMF is considerably less toxic to tumour-bearing BDF_1 mice than NMF and, unlike NMF, does not reduce hepatic glutathione levels *in vivo*. HMF has no inhibitory activity against the TLX5 lymphoma or the Sarcoma 180 in mice *in vivo* and only marginal activity against the M5076 reticulum cell sarcoma; these tumours are highly sensitive to NMF. However, like NMF, HMF inhibits growth of the human mammary tumour MX-1 implanted in the subrenal capsule of mice.

N-Methylformamide (NMF; NSC 3051) was first reported to be active against murine tumours in 1953 [1] and was given a limited phase 1 trial in 1956 [2]: the study was terminated when indications of hepatotoxicity emerged. Recently NMF has demonstrated pronounced activity against human tumour lines MX-1, LX-1 and CX-1 grown as xenografts in mice [3] and its clinical potential is being re-evaluated.

Preliminary results in man (J. G. McVie and E. Newlands, personal communication) indicate that the hepatotoxicity of the drug can be minimized by manipulating the scheduling of the drug, and that NMF, apparently, has no hematopoetic toxicity. Similar conclusions were drawn from a toxicological investigation of NMF in the dog [4].

A recent re-investigation of the structure–antitumour activity relationships between NMF and related formamides confirmed the requirement for an *N*-methyl group for optimum antineoplastic activity *in vivo* in mice [5]. Moreover, a preliminary comparison of NMF cytotoxicity *in vivo* and *in vitro* pointed to the conclusion that NMF may require metabolic activation by the host [5]. Indirect evidence suggests that *N*-hydroxymethylformamide (HMF) (Fig. 1) could be a urinary metabolite of NMF in the mouse since it is presumably the precursor of the formamide identified (GLC) in the urine of NMF-treated mice [6].

The *in vivo* antitumour activity of a number of drugs which, like NMF, have a strict structural requirement for an *N*-methyl group has been ascribed, tentatively, by us [7–9] to the formation of 'stable' *N*-hydroxymethylmetabolites which may either deposit cytotoxic formaldehyde *in situ* or react covalently as intact moieties with target bionucleophiles in a Mannich-type reaction. HMF is an attractive candidate for the active antitumour species liberated from NMF *in vivo* and in this study we have measured HMF cytotoxicity *in vitro*, its antitumour properties *in vivo* against a range of tumour systems, and have attempted to evaluate the contribution that released formaldehyde makes to the overall effects.

MATERIALS AND METHODS

Drugs and reagents. *N*-Hydroxymethylformamide was prepared by heating formamide and paraformaldehyde in the presence of sodium hydroxide at 90–100° according to the method of Grady and Stott [10]. The colourless viscous liquid (found: C, 31.73; H, 6.84; N, 18.22. $C_2H_5NO_2$ requires: C, 32.00; H, 6.71; N, 18.66%) was clarified by filtration through diatomaceous earth and used without further purification.

N-Methylformamide, formamide and 3-methyl-2-benzothiazolone hydrazone were purchased from Aldrich Chemicals (U.K.). The *N*-methylformamide was purified by redistillation at 60–62° (2 mm Hg). All other chemicals were purchased from commercial sources and were of reagent quality. Radiochemicals were purchased from Amersham International (U.K.) Ltd.: sodium [^{14}C]formate (54–58.6 mCi/mmole); DL-1-[^{14}C]leucine (59 mCi/mmole); [5-^{3}H]uridine (30 mCi/mmole); and [Me-^{3}H]thymidine (44 mCi/mmole).

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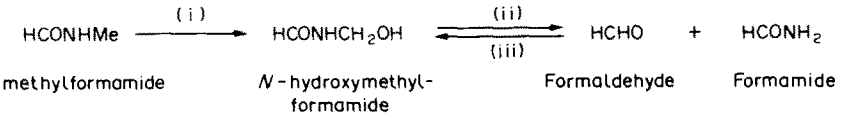

Fig. 1. Metabolic and chemical transformations of formamide derivatives. (i) Metabolic *C*-hydroxylation of *N*-methylformamide; (ii) chemical degradation of *N*-hydroxymethylformamide; (iii) chemical synthesis of *N*-hydroxymethylformamide.

Determination of formaldehyde release from N-*hydroxymethylformamide.* Solutions of *N*-hydroxymethylformamide (2.5 mM) in either normal saline, HEPES buffer (pH 7.4) or Earl's buffer (pH 7.4) were incubated at 37°. Aliquots were removed at various times and the free formaldehyde content of solutions was measured by the method of Sawicki *et al.* [11]. *N*-Hydroxymethylformamide itself was stable under the assay conditions.

Animals and tumours. The TLX5 lymphoma was passaged at 7 day intervals by transference of approximately 10^5 peritoneal ascites cells from and to CBA mice (18–21 g) as described before [5]. The M5076 reticulum cell sarcoma was passaged and grown as a solid subcutaneous tumour in female BDF_1 mice (18–23 g) as described previously [5].

The results of the antitumour tests on the MX-1 xenograft (Table 4) were supplied by the Division of Cancer Treatment, NCI, Bethesda, MD, U.S.A.

In vitro–in vivo *bioassay of drugs against TLX5 lymphoma.* Details of this procedure were as described previously [5]. In semicarbazide protection experiments TLX5 lymphoma cells were preincubated with semicarbazide (25 mM) for 1 hr.

Effects of compounds on radiolabelled precursor incorporation. TLX5 lymphoma cells were harvested from the peritoneum of male CBA mice (18–22 g) on the 6th–8th day after passage and washed with saline, then cell lysis medium [12]. Lymphoma cells (10^6) were suspended in a mixture of horse serum (4 parts) and RPMI 1640 medium (6 parts) (Gibco, Glasgow, U.K.). Aliquots of the cell suspension (15 ml) were placed in universal tubes (Sterilin, U.K.) and gassed with 10% CO_2–90% air for 1 min. After incubating for 15 min, agents were added to give the following concentrations: HMF 2.5 mM and formaldehyde 0.33 mM. In semicarbazide protection experiments, aliquots of the cell suspension were preincubated with semicarbazide (2.5 mM) for 1 hr prior to treatment with the agents. After 2 hr incubation with HMF and formaldehyde, with shaking at 37°, radiolabelled compounds were added to give a final activity of 3 μCi/ml. Samples (1 ml) were removed in triplicate at given times, placed on Whatman GF/C glass fibre filters and washed with normal saline (15 ml), ice-cold 0.2 N perchloric acid (15 ml) and then again with normal saline (15 ml). Filters were dried and counted using a Packard Tri-Carb 2660 scintillation counter with a scintillant consisting of 2,5-diphenyloxazole (5 g) and 1,4-bis(5-phenyloxazol-2-yl)benzene (0.2 g) in toluene (1l.).

Determination of hepatic non-protein thiol concentrations in BALB/c mice. These were measured as described in detail by us previously [5].

In vivo *antitumour tests.* These tests against the TLX5 lymphoma in CBA/CA mice and the Sarcoma 180 and M5076 reticulum cell sarcoma in BDF_1 mice were conducted as described previously [5].

RESULTS AND DISCUSSION

Stability of N-*hydroxymethylformamide (HMF)*

The synthetic route adopted for the preparation of HMF involved heating formamide and paraformaldehyde with sodium hydroxide [10] (Fig. 1). This method is claimed to afford a 'practically pure product' and the sample used in this study analysed satisfactorily for carbon, hydrogen and nitrogen. The IR spectrum of the colourless, viscous liquid exhibited absorptions at 3300 (bonded NH and OH), 2880 (C–H), 1675 (amide I), 1540 (amide II) and 1040 cm^{-1} (OH) as expected. However the 220 MHz ^1H NMR spectrum gave a multiplicity of peaks between δ 8.0 and 8.4 in the region of the formyl proton absorption, suggesting that the HMF either existed in tautomeric or rotameric modifications or was contaminated with products bearing C–H groups in a similar molecular environment (possibly formaldehyde and formamide). Because HMF is thermally unstable and dissociates to starting materials and unidentified products upon attempted distillation, it cannot be purified conveniently. Freshly synthesized samples of HMF were stable and two different batches contained <1% w/w of free formaldehyde when assayed by the Nash method [13]. Stock solutions of aqueous 50% w/v HMF were most stable in the pH range 5–6 and typically contained ~0.5% of free formaldehyde; the formaldehyde was liberated as the pH increased over 8 or when stock solutions were further diluted with water. Thus a 1% w/v aqueous solution of HMF liberated ~20% of its formaldehyde at pH 6 and 37° and

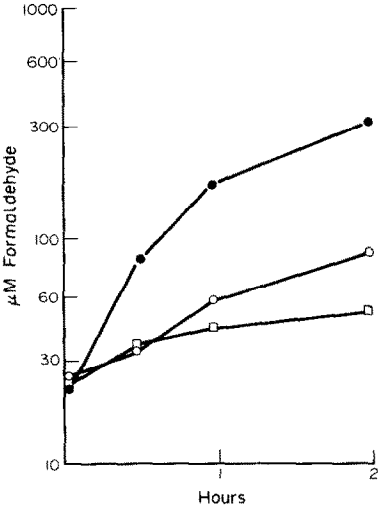

Fig. 2. The decomposition of *N*-hydroxymethylformamide (2.5 mM) at 37° to formaldehyde. (□) Physiological saline; (○) 25 mM HEPES at pH 7.4; (●) Earls buffer at pH 7.4.

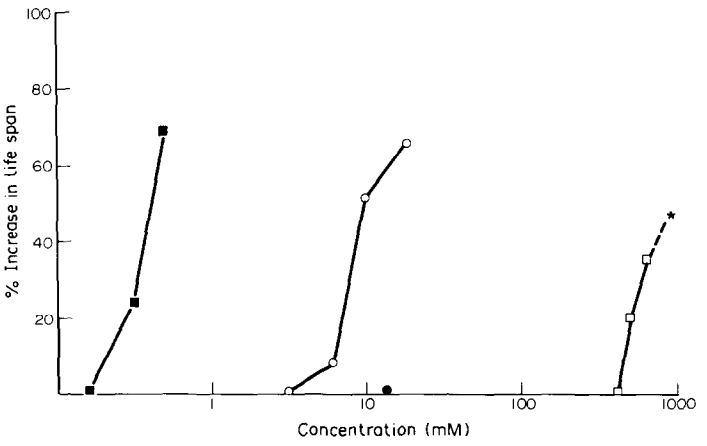

Fig. 3. Results of *in vitro–in vivo* bioassay against the TLX5 lymphoma to compare the *in vitro* cytotoxicities of formaldehyde (■), *N*-hydroxymethylformamide (○), *N*-methylformamide (□) and *N*-hydroxymethylformamide (15 mM) plus semicarbazide (25 mM) (●). Semicarbazide was without toxicity at this concentration. *Three out of five animals showed >200% increase in life span.

~95% of formaldehyde at pH 9. HMF was considerably more stable in physiological saline, Earls buffer (pH 7.4) and HEPES buffer (pH 7.4) at 37° when measured by the method of Sawicki *et al.* [11] (Fig. 2) but, again, progressive elimination of formaldehyde occurred over a 2 hr incubation period.

Thus the HMF solutions used in the following studies undoubtedly, but unavoidably, contained variable amounts of free formaldehyde, and interpretation of the results of the *in vitro* and *in vivo* experiments have been complicated by this problem. Concomitant with the liberation of formaldehyde from HMF is the production of a stoichiometric equivalent of formamide (Fig. 1) and this poses an additional problem since whereas formaldehyde is particularly active in *in vitro* cytoxicity tests, formamide exhibits modest *in vivo* activity against certain murine tumours [5].

In vitro *studies*

A comparison of the *in vitro* cytotoxicity of HMF, NMF and formaldehyde against TLX5 lymphoma cells using the bioassay technique established that HMF was an order of magnitude more cytotoxic than NMF (Fig. 3). The cytotoxicity elicited by 15 mM HMF was equivalent to that produced by 420 μM of formaldehyde: this would be equivalent to a 2.8% release of formaldehyde from HMF assuming all of the HMF cytotoxicity to be caused by free formaldehyde. Moreover, the cytotoxicity of HMF (15 mM) was abolished when TLX5 lymphoma cells were preincubated for 1 hr with semicarbazide (25 mM), a concentration which itself demonstrated no toxicity under bioassay conditions (Fig. 3). Semicarbazide has been used previously to assess the relative cytotoxicities of free formaldehyde and *N*-hydroxymethylpentamethylmelamine to PC6 cells [14]. The semicarbazide protected the cells against the former, but not the latter, and this result was interpreted as confirming that this *N*-hydroxymethyl derivative, a metabolite of the antitumour drug hexamethylmelamine [15], has cytotoxic activity *per se* against this tumour cell line. In contrast, semicarbazide could completely abolish the cytotoxicity of *N*-hydroxymethylpentamethylmelamine to L1210 and TLX5 cells *in vitro* [14]. Our experience with HMF is comparable to the latter results* and points to formaldehyde being the species responsible for cytotoxicity against the TLX5 lymphoma. The chemical nature of the semicarbazide protection is unclear: it is not simply a matter of formaldehyde semicarbazone formation because this derivative cannot be prepared from semicarbazide and aqueous formaldehyde [16].

At a concentration of 30 mM, HMF completely inhibited the incorporation of radiolabelled thymidine, uridine, leucine and formate into acid-insoluble macromolecules of TLX5 lymphoma cells. NMF did not interfere with precursor incorporation at 30 mM concentration. In the presence of HMF (2.5 mM) incorporation was as shown in Fig. 4. Preincubation of TLX5 lymphoma cells with semicarbazide (2.5 mM) for 1 hr abolished the reduction of incorporation of [5-^{3}H]uridine (Fig. 5): semicarbazide also afforded complete protection to the cells from the effects of free formaldehyde.

In vivo *studies*

Whereas formamide, NMF and *N*-ethylformamide (NEF) are of comparable toxicity to tumour-bearing BDF_1 mice when given over a 17 day schedule (Table 1), HMF is markedly less toxic. *N,N*-Dimethylformamide (DMF) is of intermediate toxicity. It has been suggested, speculatively, that the hepatotoxicity of NMF might be associated with its ability to depress hepatic non-protein thiols [5]. Thus NMF administered as a single i.p. dose of 400 mg/kg produced a dramatic reduction in liver thiols (in BALB/c mice) (Fig. 6). Because this effect can be abolished by pretreatment of the animals with SKF 525A [5], a reactive—presumably electrophilic—metabolite of NMF may be implicated. This is evidently not HMF since this hydroxylated derivative has no effect on liver non-protein thiols (Fig. 6).

* We thank a referee for bringing this distinction to our attention.

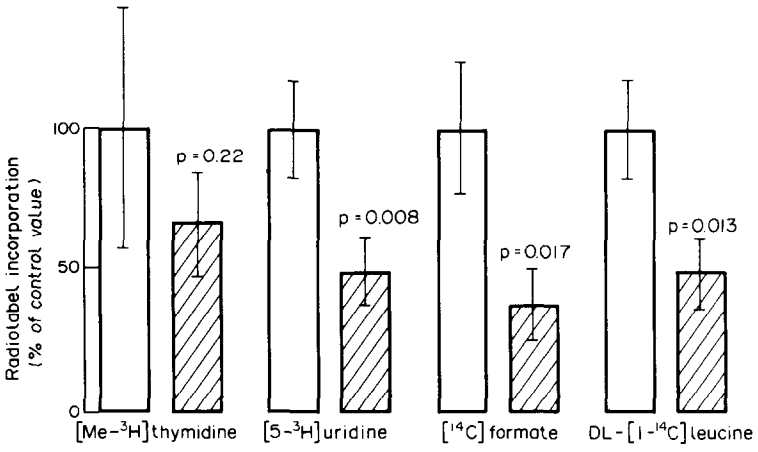

Fig. 4. The effect of *N*-hydroxymethylformamide (2.5 mM) on the incorporation of radiolabelled precursors into TLX5 lymphoma cell macromolecules after 2 hr incubation at 37°. Results are the mean ± S.E.M. of three experiments and are expressed as a percentage of the control incorporation after 1 hr incubation with the radiolabelled biomolecules. Open bars: controls; hatched bars: treated cells.

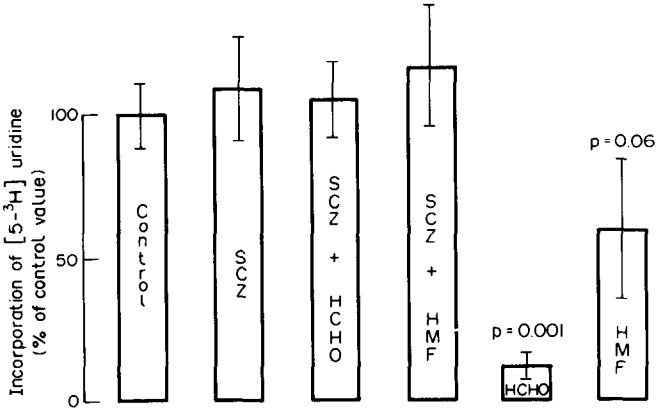

Fig. 5. The effect of preincubation of TLX5 lymphoma cells with semicarbazide (SCZ) (2.5 mM) before the addition of *N*-hydroxymethylformamide (HMF) (2.5 mM) or formaldehyde (HCHO) (0.33 mM) on the incorporation of [5-^{3}H]uridine into macromolecules ($n = 3 \pm$ S.E.M.).

Table 1. Toxicity of formamide derivatives to tumour bearing BDF_1 mice

	Treatment schedule			
	Days 1–9		Days 1–17	
Compound	LD_{10}*	LD_{50}*	LD_{10}*	LD_{50}*
Formamide	320	400	200	270
N-methylformamide	312	378	220	300
N-ethylformamide	415	490	320	420
N,N-dimethylformamide	1080	1230	1130	1280
N-hydroxymethylformamide	>1500	>1500	1580	1930

Protocol: Formamide derivatives were injected i.p. daily to female BDF_1 mice bearing either the M5076 sarcoma (days 1–17) or the sarcoma 180 (days 1–9). Groups of ten mice were used for each dose ranging from non-lethal to lethal and animals were observed until day 24. A graph of mortality vs log dose was plotted and LD_{10} and LD_{50} doses estimated from lines of best fit.

* Expressed as mg/kg per day.

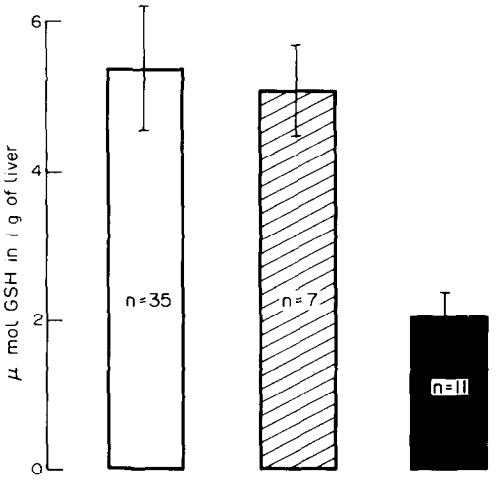

Fig. 6. The effect of *N*-hydroxymethylformamide (hatched bar) and *N*-methylformamide (closed bar) (dose: 400 mg/kg by i.p. injection) on the concentration of non-protein thiols (GSH) in livers of male BALB/c mice. Open bar: control GSH levels.

Although HMF preparations are more cytotoxic than NMF to TLX5 lymphoma cells *in vitro*, the effects *in vivo* are reversed. Thus NMF can produce significant increases in the life span of mice bearing this tumour (Fig. 7): HMF, on the other hand, is without effect. Formamide is also inactive against this tumour model [5].

The formamide derivatives were also tested by i.p. injection against the i.m. implanted Sarcoma 180 in mice. NMF and formamide produced a modest degree of activity, whereas DMF, NEF, HMF and formaldehyde were all inactive (Table 2). The results corroborate those of Clarke *et al.* [1] and confirm the importance of the *N*-methyl moiety in this series of compounds. It could be argued that the inactivity of HMF against distant tumours when administered by the i.p. route is not proof that HMF is not the active metabolite of NMF and that the potential effectiveness of the agent is thwarted by unfavourable pharmacokinetic factors. This is unlikely, however, bearing in mind the use of HMF in the foregoing antitumour tests in the intensive schedules close to the limits of host toxicity.

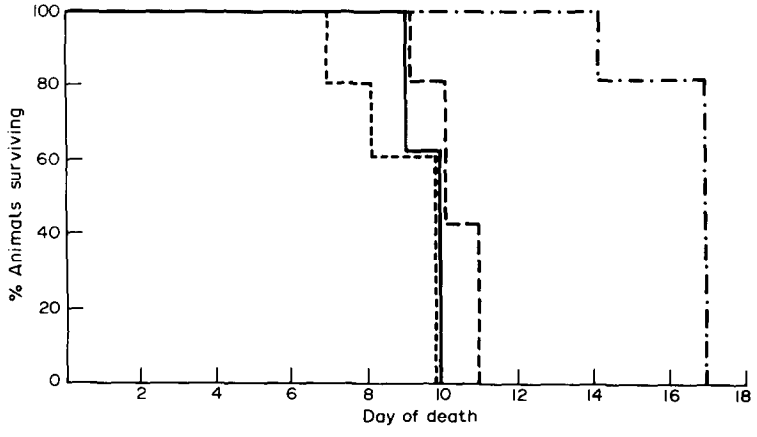

Fig. 7. *In vivo* antitumour effects of *N*-hydroxymethylformamide (HMF) and *N*-methylformamide (NMF) against the TLX5 lymphoma in mice utilizing the standard survival time assay [5]. —— Controls, ----- HMF at 1600 mg/kg by i.p. injection (daily on days 3–7 inclusive). – – – HMF at 1600 mg/kg by i.p. injection (twice daily on days 1–7 inclusive). –·–·– NMF at 400 mg/kg by i.p. injection (daily on days 3–7 inclusive).

Table 2. The antitumour activity of formamide derivatives and formaldehyde against Sarcoma 180

Compound	Dose (mg/kg per day)	% Inhibition			
		Day 6	Day 10	Day 13	Day 16
Formamide	300	41	48	55	47
N-methylformamide	300	>64	76	65	52
N-ethylformamide	300	24	20	20	11
N,*N*-dimethylformamide	1000	31	13	12	0
	1500	21	21	26	23
N-hydroxymethylformamide	1000	34	13	17	4
Formaldehyde	25*	0	3	3	10

Protocol: 10^6 tumour cells were implanted i.m. in the rear right leg of groups of ten BDF_1 mice on day 0. Drugs were administered i.p. on days 1–9 and tumour volumes were measured by calipers on days 6, 10, 13 and 16 after implantation.

* Drug administered twice daily for nine days.

The M5076 reticulum cell sarcoma is more sensitive than the TLX5 lymphoma and Sarcoma 180 to the formamide derivatives. Against this tumour, HMF, formamide and DMF were capable of eliciting inhibition of tumour growth albeit at near-toxic dose schedules. However, NMF was clearly superior to all the analogues in this test (Table 3).

Interestingly, preparations of HMF show pronounced activity against the subrenal capsule-implanted human MX-1 breast xenograft growing in athymic mice (Table 4), and the activity of HMF against human cell lines is the subject of further study.

In summary, the results presented here do not support the hypothesis that HMF is the active metabolite of NMF in murine tumour systems. Thus although HMF is more cytotoxic to TLX5 lymphoma cells than NMF *in vitro* (Fig. 3) and, unlike NMF, inhibits the incorporation of radiolabelled precursors into acid-insoluble macromolecules into the cells at 2.5 mM concentration (Fig. 4), the effects appear to be mediated by formaldehyde release alone, since they can be blocked by semicarbazide. Whereas HMF is inactive against the TLX5 lymphoma and Sarcoma 180 *in vivo*, and has only marginal activity against the M5076 reticulum cell sarcoma, NMF, on the other hand, shows pronounced activity against all three tumour lines. Moreover, the lack of effect of HMF on hepatic non-protein thiols (Fig. 6) strongly suggests that a reactive metabolite of NMF, other than HMF, may be responsible for this depletion and possible for its *in vivo* antitumour effects also.

Acknowledgements—We thank the Cancer Research Campaign for financial support, Mr. P. Behan for technical assistance with the animal studies, and Dr. G. Atassi of the Institut Jules Bordet in Brussels for providing facilities for antitumour testing.

Table 3. Antitumour activity of formamide derivatives against M5076 reticulum cell sarcoma

Compound	Optimum dose (mg/kg per day)*	% Inhibition (on day 24)
Formamide	200	63
N-methylformamide	200	100
N-ethylformamide	300	22
N,*N*-dimethylformamide	1000	60
N-hydroxymethylformamide	1500	63

Protocol: The M5076 was passaged and grown as a solid subcutaneous tumour in female BDF_1 mice (18–23 g) as described previously [5]. Drugs were administered i.p. on days 1–17 and tumour volumes were measured by calipers on day 24 after implantation.

* The optimum dose is the dose nearest in value to the LD_{10} dose.

Table 4. Activity of *N*-hydroxymethylformamide* against the subrenal capsule implanted MX-1 breast xenograft†

Host	Dose (mg/kg per day)	Survivors (test/control)	Wt. difference (test/control)	Evaluation		*T*/*C* (%)
				Test	Control	
Nu/Nu Swiss athymic mouse	2000	05/06	−1.0	0.48	9.60	5
	1200	06/06	−2.2	3.57	9.60	37
	600	06/06	−1.0	5.94	9.60	62
	300	06/06	−0.7	10.63	9.60	111
Nu/Nu BALB/c athymic mouse	2000	06/06	−4.5	1.37	17.19	8
	1200	06/06	−3.1	1.82	17.19	11
	600	06/06	−1.2	6.65	17.19	39
	300	06/06	−0.4	11.62	17.19	68

* Also designated NSC 348403.

† For details of protocol see [17]. In brief, fragments of the MX-1 breast xenograft were implanted under the renal capsule of recipient mice on day 0. The tumour length (a) and width (b) were measured *in situ* in ocular micrometer units (OMU). HMF was administered daily between days 1 and 10 post tumour implant. On day 11 the tumour was again measured and activity was based on the change in average tumour diameter over the prescribed course of treatment compared with the change in average control diameter. Thus $T/C\ (\%) = DT/DC \times 100$ where DT is the mean tumour diameter $(a + b)/2$ of the treated group at the end of treatment less the mean tumour diameter at the beginning of treatment and where DC is the change in mean tumour diameter of controls over the same period.

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