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Pharmacokinetics and biochemistry studies on nicotinamide in the mouse

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Abstract. Nicotinamide sensitizes murine tumours to the effect of radiation, but the pharmacokinetics are not well characterized at doses that are achievable in humans. In the mouse, nicotinamide given i.p. at doses of 100–500 mg/kg showed biphasic elimination with dose-dependent changes in half-life. The initial half-life increased significantly ($P < 0.05$) from 0.8 to 2 h and the terminal half-life increased from 3.4 to 5.6 h over the dose range studied. Clearance, however, decreased significantly from 0.3 to 0.24 l kg^{-1} h^{-1} only at the highest dose. Peak concentrations increased in a dose-dependent manner from 1,000 to 4,800 nmol/ml. The main plasma metabolite in the mouse is nicotinamide *N*-oxide, the peak concentration of which increased only from 80 to 160 nmol/ml. The *N*-oxide, which is also a weak radiosensitizer, is subject to reduction to the parent nicotinamide following administration at a dose of 276 mg/kg; peak concentrations of the *N*-oxide of 1900 nmol/ml were reached in 10 min, whereas concentrations of nicotinamide produced by reduction reached a maximum of 144 nmol/ml at 1 h. Elimination of the *N*-oxide was also biphasic, with initial and terminal half-lives being 0.39 and 1.8 h, respectively. The bioavailability of both drugs given via the i.p. as compared with the i.v. route was close to 100%. Tumour concentrations of nicotinamide paralleled those in the plasma after a short lag. Tumour nicotinamide adenine dinucleotide (NAD) concentrations were elevated by factors of 1.5 and 1.8 following doses of 100 and 500 mg/kg nicotinamide, respectively. Maximal concentrations were seen after 3–6 h, but levels remained elevated for 16 h. No change in tumour energy charge or in plasma 5-hydroxytryptamine was detected following a dose of 500 mg/kg nicotinamide.

Key words: Nicotinamide – Nicotinamide *N*-oxide – Pharmacokinetics

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Introduction

Nicotinamide is known to sensitize a number of rodent tumours to single doses of radiation [8], whereas the combination of nicotinamide with carbogen (95% oxygen, 5% carbon dioxide) results in a large enhancement of tumour response in fractionated regimes [12], and the combination is currently beginning clinical trials. Large doses of up to 12 g daily have been tolerated in patients over many months [7, 27], and the pharmacokinetics in humans suggest that sufficient drug can be given to achieve radiosensitization [19, 24]. However, little is known about the fundamental mechanism of action of nicotinamide, although it appears to lead to a reduction in the number of acutely hypoxic cells [6, 10] and to a lowering of tumour interstitial fluid pressure [13]. It is ineffective as a radiosensitizer in vitro, which suggests a physiological mechanism, but it is possible that there could be a component of its activity that requires metabolic conversion to an active species; at least eight products have been identified in a number of different species, including humans [4, 5, 14–16].

There are relatively few data available on the metabolism and pharmacokinetics of nicotinamide in the mouse; Horsman et al. [8] measured plasma and tumour nicotinamide concentrations after doses of 1,000 mg/kg, and several studies have compared urinary excretion of nicotinamide and its metabolites in a number of rodent species [20]. A number of analogues of nicotinamide have also been studied that show some radiosensitizing activity in vivo [2], as does the metabolite nicotinamide *N*-oxide (M. R. Horsman, personal communication), which is known to be capable of substituting for nicotinamide in the rat [21].

Nicotinamide is also involved in anabolic reactions, including the biosynthesis of nicotinamide adenine dinucleotide (NAD), which is known to decrease markedly following irradiation [3]. This is due to the requirement of NAD in the repair of radiation-induced damage via the synthesis of polyADP-ribose by the enzyme adenosine diphosphate (ADP)-ribosyl transferase [22]. Nicotinamide

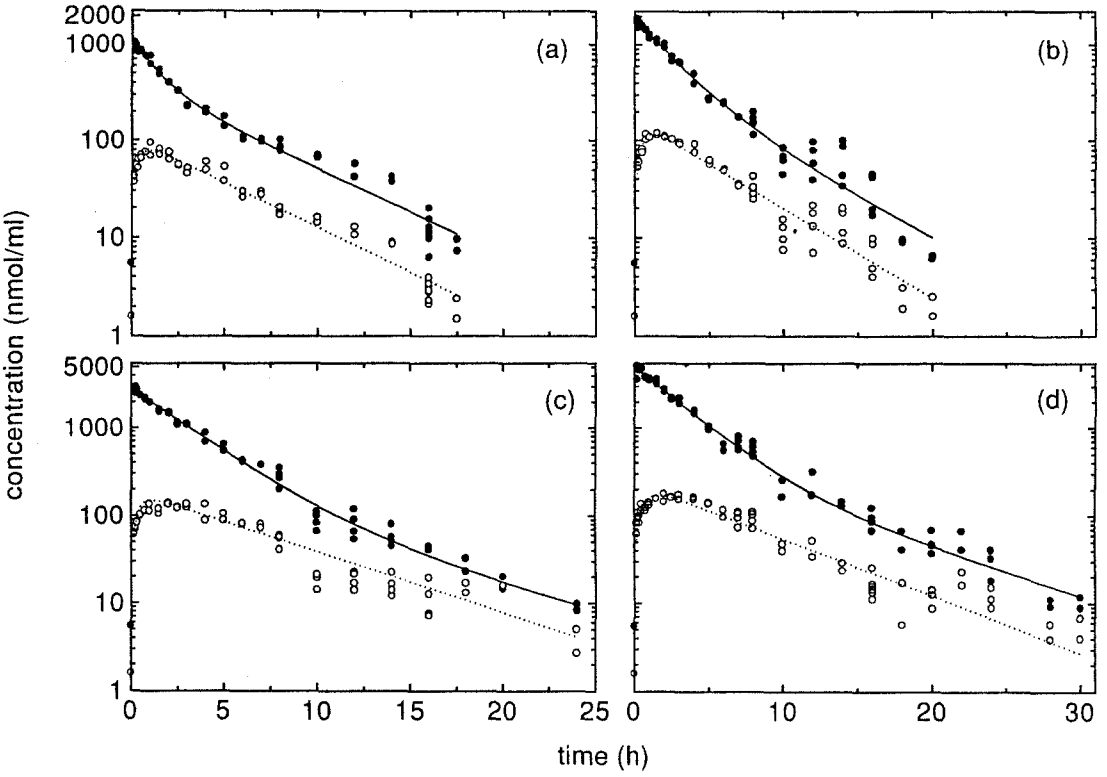

Fig. 1a–d. Plasma concentrations of nicotinamide (●) and nicotinamide *N*-oxide (○) obtained after i.p. administration of nicotinamide at **a** 100 mg/kg, **b** 200 mg/kg, **c** 300 mg/kg and **d** 500 mg/kg

inhibits the enzyme in vitro, although this occurs only at very high concentrations, and it seems unlikely that this biochemical pathway would be involved in nicotinamide radiosensitization in vivo. Nicotinamide is also known to be an inhibitor of tryptophan pyrrolase [26], the first enzyme in the complex pathway that converts tryptophan to NAD; it has been suggested that this inhibition may increase the conversion of tryptophan to the powerful vasoactive compound 5-hydroxytryptamine (5-HT, serotonin). Finally, nicotinamide administration results in an increase in tumour energy charge [25], which may reflect the improved oxygen supply to the tumour or an interaction with other aspects of intermediary metabolism.

Because of these questions concerning the mechanism of action of nicotinamide and the complexity of its metabolism, we studied the pharmacokinetics and metabolism of nicotinamide and its *N*-oxide in the mouse.

Materials and methods

Mice and tumours. Animals were 10- to 15-week-old males (strain CBA/Ht/GyfBSVS). Tumours were implanted s.c. on the back with the carcinoma NT (CaNT) and were used with diameters of 7–10 mm. Nicotinamide (Merck) was dissolved in saline such that the volume delivered was always 0.01 ml/g. Because of the lower solubility of nicotinamide *N*-oxide (Sigma), the volumes given were 0.02 (i.p.) and 0.0125 ml/g (i.v.).

Drug analysis. For measurement of drug concentrations, mice were killed by decapitation and the blood was collected into heparinized tubes. The blood was cooled on ice and centrifuged at 4° C, and the plasma was separated off. Samples were stored at –20° C prior to analysis of nicotinamide and its metabolites by high-performance liquid chromatography (HPLC) as previously described [23]. For tumour measurements, mice were killed by neck luxation, and the tumour and surrounding skin were immediately removed and clamped between two aluminium plates cooled in liquid nitrogen. The frozen tissue was ground to a powder in a pestle and mortar in liquid nitrogen, allowing the removal of attached skin that remained in sheets. The tumour tissue was then quickly weighed and homogenized in ice-cold 100 m*M* hydrochloric acid. An aliquot of this was then analyzed as described for plasma, and the remainder was stored at –70° C for nucleotide analysis. The dilute HCl/methanol extraction technique was used in preference to other acids such as perchloric or trichloroacetic acid because these were not compatible with the chromatographic methods used. Some acid was necessary to prevent enzymic degradation of NAD and adenosine triphosphate (ATP) during extraction.

For the measurement of free plasma 5-HT, it was necessary to give the mice heparin (2000 units) 30 min prior to euthanasia and to anaesthetize the mice with Metofane at the time of sampling; the chest wall was opened, the blood vessels from the heart were cut, and blood was collected immediately into a tube cooled on ice. The blood was centrifuged at 9,500 *g* for 2 min and an aliquot of the plasma was immediately deproteinized with 4% trichloroacetic acid. After centrifugation to remove the precipitate, the supernatant was analyzed for 5-HT by HPLC using a pre-column sample-enrichment technique similar to that of Palmerini et al. [18], except that a reversed-phase C18 analytical column was used (Hypersil 5ODS, 250×4.6 mm, Hichrom Ltd.), and the eluent was similar to that employed by Munoz et al. [17].

Tumour adenine nucleotides were determined in the freeze-clamped acid extracts using a post-column derivatization technique employing chloroacetaldehyde. Full details of the method will be published elsewhere.

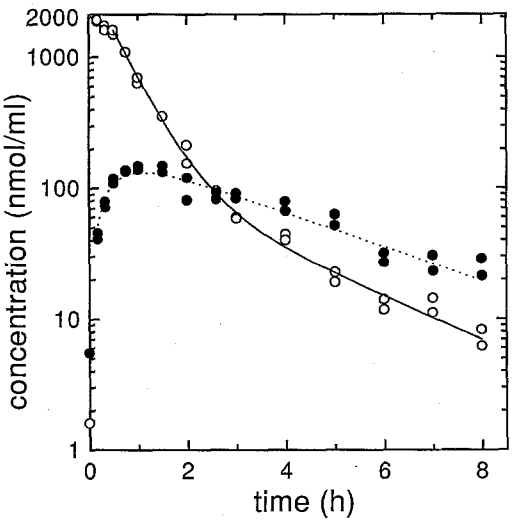

Fig. 2. Plasma concentrations of nicotinamide (●) and nicotinamide *N*-oxide (○) obtained after i.p. administration of nicotinamide *N*-oxide at 276 mg/kg

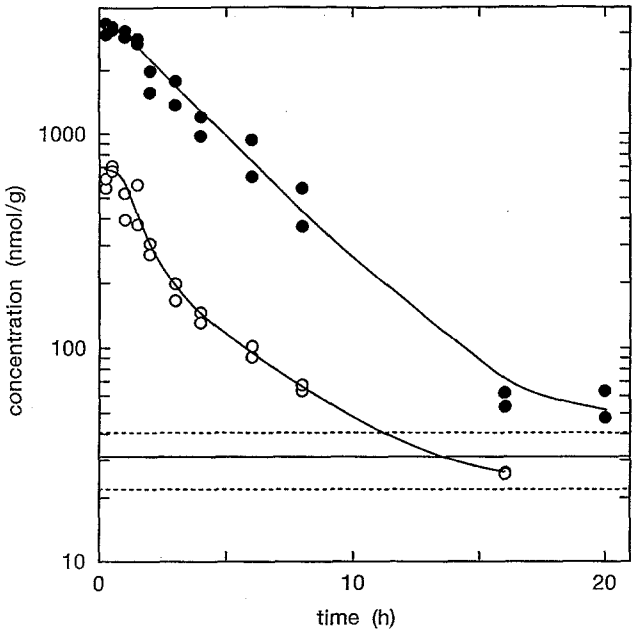

Fig. 3. Tumour concentrations of nicotinamide obtained after i.p. administration of 100 mg/kg (○) and 500 mg/kg (●). The *solid and dotted lines* represent the control value and 95% confidence limits, respectively

Pharmacokinetic analysis. Using a computer-based non-linear least-squares program (Origin, MicroCal Software Inc.), the plasma concentration-time profiles were fitted to the following equation:

$$C_t = Ae^{-\alpha t} + Be^{-\beta t}.$$

The area under the curve (AUC) was calculated by summing the area to the latest time (t) using the trapezium rule; where the plasma levels did not reach those achieved in control mice, the time at which this would have occurred was extrapolated from β and the additional area was calculated as C_t/β, where C_t was the concentration at the last time point measured. C_0 was calculated as $A + B$ and the volume of distribution of the central compartment (V_c), as $Dose/C_0$. Clearance was calculated as $Dose/AUC$, assuming complete drug absorption.

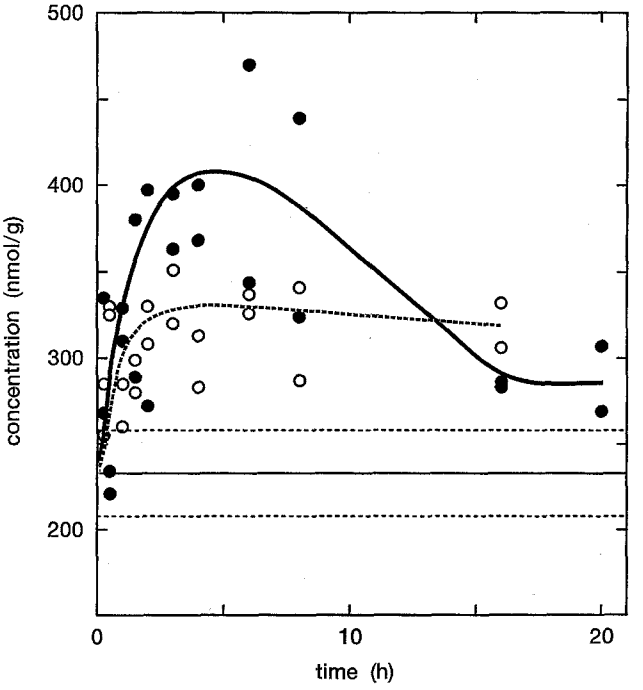

Fig. 4. Tumour concentrations of NAD obtained after i.p. administration of nicotinamide at 100 mg/kg (- - ○ - -) and 500 mg/kg (—●—). The *horizontal solid and dotted lines* represent the control value and 95% confidence limits, respectively

Results

Plasma concentrations of nicotinamide and the *N*-oxide resulting from i.p. administration of 100, 200, 300 and 500 mg/kg nicotinamide are shown in Fig. 1, and those obtained using 276 mg/kg nicotinamide *N*-oxide are presented in Fig. 2. Data recorded following i.v. administration of the lowest and highest doses of nicotinamide and of the single dose of its *N*-oxide were also obtained up to 8 h, and the AUCs calculated are shown in Table 1. Nicotinamide and nicotinamide *N*-oxide were present in control mice at concentrations of 5.5±0.7 (SE, n = 11) and 1.6±0.1 (SE, n = 11) nmol/ml, respectively. No significant amount of any other metabolite was detected for either drug. The initial slopes of the clearance curves for nicotinamide appeared similar, but use of the principle of superposition, dividing the concentration by the dose, gave curves suggesting that clearance was slightly dose-dependent over this range of doses (data not shown). The later-time samples showed the presence of biphasic clearance with dose-dependent changes in values for α and β elimination rate constants, both of which increased by a factor of

Table 1. Bioavailability of nicotinamide and nicotinamide *N*-oxide

Drug	Dose (mg/kg)	$AUC_{0-8\,h}$ (nmol ml^{-1} h) ± SE	
		i.v.	i.p.
Nicotinamide	100	2,150±85	2,350±72
Nicotinamide	500	14,880±380	14,690±560
Nicotinamide *N*-oxide	276	1,660±88	1,890±58

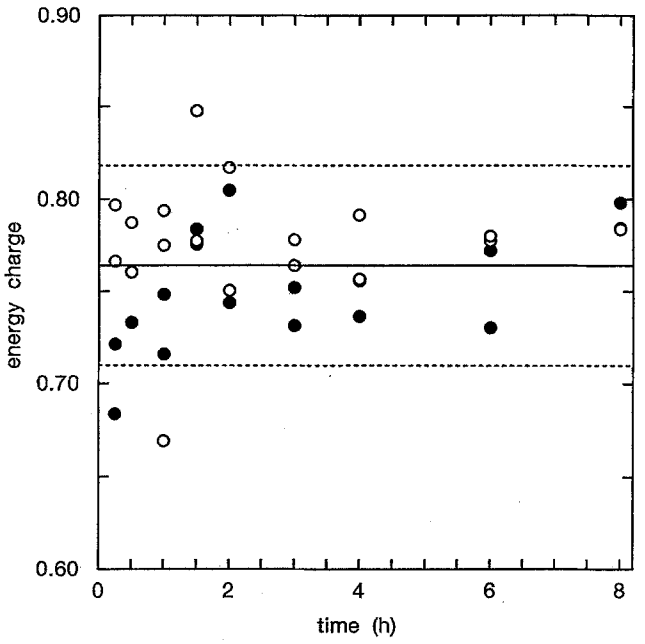

Fig. 5. Tumour energy charge determined after i.p. administration of nicotinamide at 100 mg/kg (○) and 500 mg/kg (●). The *solid and dotted lines* represent the control value and 95% confidence limits, respectively

about 2 over the dose range studied. Nicotinamide *N*-oxide was subject to reduction to the parent nicotinamide to a significant extent (Fig. 2) and also showed biphasic elimination after a slightly slower absorption phase. Pharmacokinetic parameters calculated for the doses used are shown in Table 2. In all cases, the goodness-of-fit parameter χ^2 indicated that the biexponential model provided a good fit to the data.

Tumour concentrations of nicotinamide detected following doses of 100 and 500 mg/kg are shown in Fig. 3. Although the *N*-oxide was detectable in the tumours and its concentration appeared to increase with dose, the data are not presented because in the control mice the chromatogram showed an interfering peak that made accurate quantification difficult. A comparison of these data with the corresponding plasma concentrations shown in Fig. 1 shows that nicotinamide readily penetrates tumour tissue, rapidly approaching the levels seen in plasma, as has previously been demonstrated [8]. However, control levels measured in tumour of ~30 nmol/g are much higher than those observed in the plasma. Figure 4 shows the levels of NAD measured in the freeze-clamped tumours. Although there is considerable scatter in the data, there appears to be a significant increase in NAD concentration with both doses of nicotinamide, which takes many hours to return to control levels.

Figure 5 plots the energy charge, calculated as [ATP] + 0.5[ADP]/[ATP] + [ADP] + [AMP] (adenosine mono-

Table 2. Pharmacokinetic parameters obtained after i.p. administration of nicotinamide or nicotinamide *N*-oxide. Values are given ± SE

	Dose, mg/kg (mmol/kg)				
	Nicotinamide				*N*-oxide
	100 (0.82)	200 (1.64)	300 (2.46)	500 (4.10)	276 (2.00)
A (nmol/ml)	705 ±32	1,440 ±124	2,660 ±89	4,620 ±91	3,570 ±152
α (h^{-1})	0.821 ±0.064	0.446* ±0.029	0.360* ±0.012	0.350 ±0.007	1.77 ±0.08
$t_{1/2}$ (h)	0.844 ±0.066	1.55* ±0.10	1.93* ±0.064	1.98 ±0.04	0.39 ±0.02
B (nmol/ml)	400 ±35	439 ±131	221 ±97	503 ±98	149 ±39
β (h^{-1})	0.206 ±0.009	0.190 ±0.019	0.133* ±0.024	0.125 ±0.009	0.382 ±0.051
$t_{1/2}$ (h)	3.36 ±0.15	3.65 ±0.37	5.21* ±0.94	5.55 ±0.40	1.81 ±0.24
C_0 (nmol/ml)	1,110 ±47	1,880 ±180	2,880 ±132	5,120 ±134	3,720 ±157
V_c (l/kg)	0.742 ±0.032	0.872 ±0.083	0.854 ±0.039	0.801 ±0.021	0.538 ±0.023
AUC (nmol ml^{-1} h)	2,780 ±104	5,500 ±223	8,900 ±321	17,000 ±837	1,930 ±62
Clearance (l kg^{-1} h^{-1})	0.295 ±0.011	0.298 ±0.012	0.276 ±0.010	0.241** ±0.012	1.04 ±0.033
χ^2 (*n*)	3.3 (48)	7.1 (54)	9.0 (56)	22.5 (62)	1.6 (24)

* Significantly different from the preceding dose ($P < 0.05$)
** Significantly different from 100 and 200 mg/kg

Table 3. Effect of nicotinamide (500 mg/kg) on plasma 5-HT

Time after nicotinamide (min)	5-HT (nmol/ml) ± SE
0	0.088 ± 0.041 (n = 14)
10	0.081 ± 0.043 (n = 9)
20	0.056 ± 0.024 (n = 4)

phosphate), as determined in the freeze-clamped tumour extracts. At the doses studied nicotinamide did not appear to have any significant effect on the energy charge or on the concentrations of the individual nucleotides (data not shown).

Table 3 shows the plasma concentrations of 5-HT measured at two times after nicotinamide administration. There was no significant difference between the two dose groups and the controls.

Discussion

Table 1 indicates that the bioavailability of both drugs was close to 100%; there was also no significant difference in the formation of the major metabolite (the *N*-oxide for nicotinamide and nicotinamide for the *N*-oxide) following i.v. or i.p. administration (data not shown). All further studies were carried out using the i.p. route. The biphasic clearance of nicotinamide in the mouse (Figs. 1, 2) has not been observed previously since it is seen only when plasma concentrations fall below ~200 nmol/ml; previously published data have involved higher doses and have not investigated later times. The effect seen contrasts with that observed in humans [24], where at plasma concentrations below ~100 nmol/ml, plasma clearance becomes much more rapid, with the half-life decreasing from around 8 h to less than 2 h. In humans this reduction in plasma half-life seems to coincide with a relative increase in methyl nicotinamide concentration, but in the mouse this methylation pathway does not seem to be important, as neither methyl nicotinamide nor the further oxidation products *N*-methyl-2- or 4-pyridone-5-carboxamide are seen in significant concentrations, although small amounts are detectable in mouse urine [20, 23]. This contrast between slow and fast terminal clearance in mice as compared with humans may be related to the observation that control levels of both nicotinamide and *N*-oxide are at least 1 order of magnitude lower in humans as compared with mice.

In the mouse, *N*-oxidation is a much more important metabolic pathway following nicotinamide dosing [20], although saturation of this pathway may be occurring, since the peak *N*-oxide concentration does not increase in a dose-dependent manner, increasing only from 80 to 160 nmol/ml when the nicotinamide dose is increased 5-fold. Zero-time intercepts of exponential fits to the terminal portion of the *N*-oxide profiles observed following nicotinamide dosing also increased by a factor of less than 3 over the dose range studied, whereas the slopes paralleled those of the nicotinamide, as would be expected for a more rapidly cleared polar metabolite. For the nicotinamide formed from the *N*-oxide, the terminal half-life was 2.5 h, slightly less than that seen after dosing with 100 mg/kg nicotinamide, consistent with the lower concentration seen after the *N*-oxide administration. It may be that the slow terminal clearance of nicotinamide seen after all doses was due in part to the back-conversion of the *N*-oxide to the parent drug seen after nicotinamide *N*-oxide administration (Fig. 2). There may also be release into the plasma of nicotinamide derived from catabolism of the so-called "storage NAD", which is known to be formed in the liver of rats and mice following administration of large doses of nicotinamide [1]. This may explain both the biphasic elimination seen for all drug doses, due to the slow release of further quantities of the compound at late times after drug administration, and the surprisingly small decrease in clearance with dose as compared with the apparent changes in the elimination rate constants.

The calculated volumes of distribution (Table 2) indicate that nicotinamide, despite its relatively high polarity, is quite well distributed, as is also evidenced by the good tumour concentrations observed (Fig. 3). As might be expected, the very polar *N*-oxide has a much reduced volume of distribution, whereas its clearance is much greater than that of nicotinamide.

The study using the *N*-oxide illustrates the importance of acquiring pharmacokinetic information during investigations of drug analogues; although the *N*-oxide has been shown to be a weak radiosensitizer in mice, much of this effect could be attributed to the conversion back to the parent nicotinamide. Only a careful study of the time course of radiosensitization combined with the pharmacokinetics would allow the relative contribution by the two compounds to be calculated. This may have important implications in the search for more potent analogues of nicotinamide.

The measured rise in tumour NAD concentration (Fig. 4), although rather variable, is consistent with the previously reported [3] effect of elevations of tumour levels of NAD leading to enhanced radiosensitization. However, the time scale of the change does not correlate with the time course of sensitization, since the peak does not occur until 2–4 h after nicotinamide administration, whereas sensitization is maximal within 1 h [8]. In addition, high levels of NAD persist for many hours; this parallels some of the changes in physiological function such as blood flow and arterial pressure, which also persist for several hours [9, 11].

Figure 5 shows that at the highest dose studied, nicotinamide had no effect on tumour energy status as measured by the relative amounts of the adenine nucleotides. This observation contrasts with the results obtained by Horsman et al. [11], who showed an increase in the concentration of ATP relative to that of both the other adenine nucleotides and inorganic phosphate. However, this study used a higher dose of 1,000 mg/kg nicotinamide.

The present data on plasma 5-HT concentrations measured following nicotinamide administration suggest that the inhibition of tryptophan pyrrolase is not an important mechanism in its action. Only early time points were studied since it is known that significant changes in systemic blood flow induced by nicotinamide occur within the first 30 min [9, 11], and any changes in 5-HT levels are expected to precede any physiological response. We mea-

sured only free plasma 5-HT, since this should reflect any whole-body effects through release from the platelet stores; however, if there were a localized stimulation of release within the tumour vasculature, this would not necessarily be detected in plasma.

This paper presents data on the pharmacokinetics and metabolism of nicotinamide given at doses that are relevant to its use as a radiosensitizer as well as data on possible biochemical bases for its action. It appears from this work that neither modulation of plasma 5-HT nor tumour energy metabolism is directly related to the mode of action of nicotinamide as a radiosensitizer. However, the pharmacokinetic data should provide a base against which information from clinical trials with nicotinamide can be compared.

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