Biochemical Pharmacology, Vol. 36, No. 9, pp. 1483–1487, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

TOXICITY OF 3-NITRONAPHTHALIMIDES TO V79 379A CHINESE HAMSTER CELLS

R. J. HODGKISS, R. W. MIDDLETON, M. R. L. STRATFORD and R. DEL BUONO
Cancer Research Campaign Gray Laboratory, Mount Vernon Hospital, Northwood, Middlesex, HA6 2RN, U.K.

(*Received* 16 *September* 1986; *accepted* 21 *November* 1986)

Astract—The cellular uptake and toxicity of a number of substituted 3-nitronaphthalimides was investigated. Uptake of these compounds into cells was initially rapid and reached a plateau after several hours, where in some cases intracellular concentrations were much greater than the corresponding extracellular concentrations. Little uptake was obtained, however, with a compound carrying an acidic substituent. Toxicity studies divided the compounds into two main groups; those where survival curves were convex and those where survival curves were concave. The shapes of survival curves of the latter group did not appear to reflect depletion of extracellular drug. Uptake and toxicity of different drugs were not well correlated and bioreductive metabolism of the nitro-substituent did not appear to be a major contributor to toxicity. There was no consistent differential toxicity of these drugs in aerobic and hypoxic conditions. It was concluded that the nature of the ring substituent had more effect on toxicity than the absolute concentration of the naphthalimide ring or bioreductive metabolism of the nitro-group.

There is considerable interest in novel cytotoxic compounds for potential use in cancer chemotherapy. One class of such compounds that has been investigated comprised the substituted naphthalimides, many of which have been evaluated for cytostatic and cytotoxic properties [1–3]. In addition to their cytotoxic effects on mammalian cells *in vitro*, several of these compounds have been shown to be effective anti-tumour and anti-viral agents *in vivo* [1, 2, 4, 5]. One of these compounds, mitonafide (5-nitro-2-(2-dimethylaminoethyl) - benzo[*de*]isoquinoline - 1,3 - dione hydrochloride) (NSC 300288) has been proposed as a suitable candidate for clinical trials as a chemotherapeutic agent [6], and another nitronaphthalimide (1H-benzo(*de*)isoquinoline-1,3(2H)-dione,5-amino-2-(2-dimethyl-aminoethyl) (NSC 308847) has undergone pre-clinical toxicology [7].

The mode of action of substituted naphthalimides is linked to their intercalation with DNA [8] resulting in increased melting temperatures and inhibition of DNA and RNA synthesis [1, 2]. In addition to their cytotoxic properties these compounds have been shown to be genotoxic, inducing DNA strand breaks, chromosome aberrations, sister chromatid exchanges and mutations in bacteria [6, 9, 10].

Some naphthalimides are highly fluorescent molecules. We have synthesised a number of novel nitronaphthalimides as potential fluorescent probes for hypoxic cells in tumours, and have recently reported on the use of one such compound in this application [11–13]. In this paper we report on the uptake and toxicity of a range of novel 3-nitronaphthalimides, synthesised as potential hypoxic cell probes.

* Abbreviations used: MEM, Eagle's minimal essential medium; fcs, foetal calf serum; PBS, phosphate buffered saline; DMSO, dimethyl sulphoxide.

MATERIALS AND METHODS

V79 379A Chinese hamster cells were maintained as exponentially growing suspension cultures in Eagle's Minimal Essential medium (MEM)* modified for suspension culture, with 7.5% foetal calf serum (fcs). Phosphate buffered saline (PBS) was prepared from Dulbecco 'A' tablets, Oxoid Ltd.

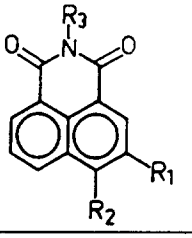
Mitonafide (Batch No. KM47-92-1) was donated by Dr V. L. Narayanan, National Cancer Institute, U.S.A. DM110 was prepared as reported in [3], DM154 and DM155 as described in [14] and DM159 as described in [15]. Synthesis of the other nitronaphthalimides will be described elsewhere.

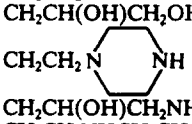
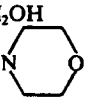
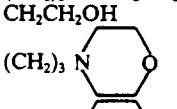
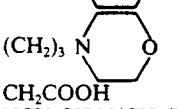
For measurements of drug uptake and toxicity, 50 cm^3 cell suspensions at 5×10^5 cells per cm^3 were stirred at 37° in 0.1 dm^3 Dreschel bottles, modified with a side arm for sampling, and gassed with 0.4 dm^3 min^{-1} air + 5% CO_2 or nitrogen + 5% CO_2 (<10 ppm O_2) obtained from the British Oxygen Co. Ltd. Cultures were pregassed for 1 hr before the addition of appropriate small volumes of drug solution. Because many of the compounds used in this study were poorly soluble in water, all drugs were initially dissolved at 10–20 mmol dm^{-3} in DMSO. The final concentration of DMSO was less than 2%.

For toxicity studies, cell samples taken through the sidearm immediately before and at various times after drug addition were centrifuged to remove the drug solution and known numbers of cells plated on 5 cm plastic petri dishes to assess viability in a 7 day colony forming assay.

For estimates of drug uptake, cell samples were centrifuged to remove drug solution, washed by resuspending in PBS and recentrifuging, and the pellet suspended in absolute alcohol and sonicated for 30 sec. The sample was centrifuged again, an

Table 1. Structures of compounds used in this paper



Compound	R_1	R_2	R_3
DM110	NO_2	H	CH_2CH_2OH
DM111	NO_2	H	$CH_2CH(OH)CH_2OH$
DM112	NO_2	H	
DM113	NO_2	H	$CH_2CH(OH)CH_2NH_2$
DM114	NO_2	H	$CH_2CH_2NHCH_2CH_2OH$
DM115	NO_2	H	$(CH_2)_3N(CH_2CH_2OH)_2$
DM118	NH_2	H	$CH_2CH(OH)CH_2NH_2$
DM124	NO_2	H	$(CH_2)_3NHCH_2CH_2CH_2NH_2$
DM154	NO_2	$NHCH_2CH_2OH$	CH_2CH_2OH
DM155	NO_2		
DM159	NO_2	H	
DM161	NO_2	H	CH_2COOH
DM163	NO_2	$NHCH_2CH_2N(CH_2CH_3)_2$	$NCH_2CH_2N(CH_2CH_3)_2$
Mitonafide	NO_2	H	$CH_2CH_2N(CH_3)_2$

aliquot removed, dried under vacuum and redissolved in eluent A. HPLC was performed using a Waters system comprising two M6000A pumps, WISP autosampler, 441 u.v. detector, 720 system controller and 730 data module. The column used was a Partisil 50DS2 (250 × 5 mm). Detection was at 340 mm except for DM154, DM155, and DM163 (405 nm). Eluents were: (A) 5 mmol dm^{-3} heptane sulphonic acid, 5 mmol dm^{-3} dibutylamine hydrochloride, 40 mmol dm^{-3} H_3PO_4, 10 mmol dm^{-3} NaH_2PO_4; (B) acetonitrile. The chromatography utilised a linear gradient from 25 to 40% B in 10 min, held for 1 min, returning to 25% at 11.5 min, except for DM155 which was run isocratically at 26% B.

RESULTS

Structures of the naphthalimides used in this work are shown in Table 1. All compounds have a 3-nitro substituent except for DM118 which has an amine group in this position. Typical survival data for DM112, DM113, DM155 and mitonafide are shown in Fig. 1, for cells incubated under oxic and hypoxic conditions. No toxicity was observed from DMSO, used as a solvent to prepare stock concentrations of drugs. Parameters of these and similar survival curves were fitted to the equation $\log(S) = -\alpha D - \beta D^2$, by the method of least-squares and used to calculate the incubation times to give 0.5 surviving fraction for each drug concentration used (a survival level of 0.5 allowed the maximum number of survival curves to be included, while the method of calculation maintains accuracy by including all the data points from each survival curve). As

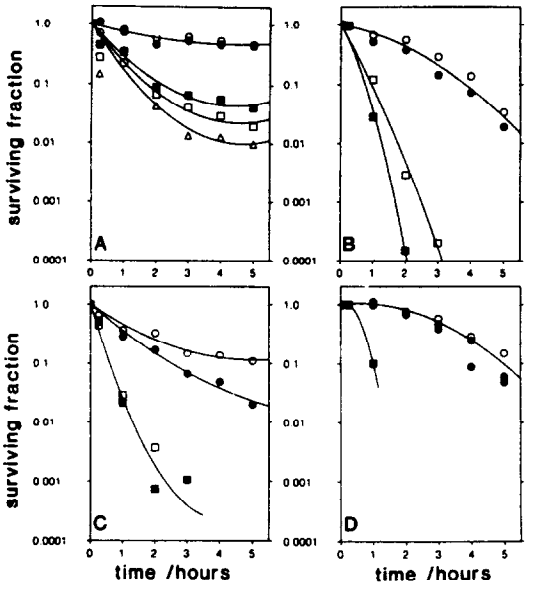


Fig. 1. Toxicity of 3-nitronaphthalimides to V79 379A Chinese hamster cells incubated with drugs in MEM + 7.5% fcs. (A) DM113 aerobic incubation: (○), 0.01 mmol dm^{-3}; (□) 0.025 mmol dm^{-3}; DM113 hypoxic incubation; (●), 0.01 mmol dm^{-3}; (■), 0.025 mmol dm^{-3}; (△), 0.05 mmol dm^{-3}. (B) DM112 aerobic incubation: (○), 0.1 mmol dm^{-3}; (□), 0.15 mmol dm^{-3}; DM112 hypoxic incubation: (●), 0.1 mmol dm^{-3}; (■), 0.15 mmol dm^{-3}. (C) Mitonafide aerobic incubation: (○), 0.5 μmol dm^{-3}; (□), 1.0 μmol dm^{-3}; mitonafide hypoxic incubation: (●), 0.5 μmol dm^{-3}; (■), 1.0 μmol dm^{-3}. (D) DM155 aerobic incubation: (○), 0.025 mmol dm^{-3}; DM155 hypoxic incubation: (●), 0.025 mmol dm^{-3}; (■), 0.05 mmol dm^{-3}.

Table 2. Uptake and toxicity of the compounds used in this paper

Compound	Uptake at* 10 μmol dm^{-3} fmol $cell^{-1}$	Uptake at* 100 μmol dm^{-3} fmol $cell^{-1}$	[Drug] /μmol dm^{-3} 0.5 S.F. 1h	n
DM161	not done	0.017	>224‡	
DM110	0.013 ± 0.002	not done†	>100†‡	
DM111	0.013 ± 0.004	0.09 ± 0.03	117 ± 36	0.28 ± 0.41
DM159	0.018 ± 0.003	0.32 ± 0.04	207 ± 24	0.58 ± 0.10
DM124	0.025 ± 0.004	0.20 ± 0.03	>150‡	
Mitonafide	0.032 ± 0.008	0.53 ± 0.13	0.46 ± 0.10	0.64 ± 0.13
DM118	0.050 ± 0.008	0.7 ± 0.03	258 ± 114	1.1 ± 0.3
DM115	0.090 ± 0.005	1.99 ± 0.05	109 ± 13	0.37 ± 0.09
DM113	0.10 ± 0.008	2.18 ± 0.07	23 ± 3	0.61 ± 0.11
DM112	0.14 ± 0.014	3.63 ± 0.17	126 ± 5	0.26 ± 0.05
DM154	0.16 ± 0.03	not done†	>100†‡	
DM114	0.21 ± 0.01	not done†	70 ± 11	0.30 ± 0.15
DM155	1.85 ± 0.10	22.3 ± 1.2	44 ± 9	0.74 ± 0.13
DM163	4.01 ± 0.71	7.08 ± 0.66	12 ± 5	0.82 ± 0.14

* Uptake measured 4 hr after drug addition, in V79 379A Chinese hamster cells, suspended in aerated MEM + 7.5%fcs. Assuming a cellular diameter of 12 μm, 1 fmol $cell^{-1}$ corresponds to an intracellular concentration of 1.1 mmol dm^{-3}.

† Limited by solubility.

‡ No toxicity; maximum concentration used.

there was no significant difference in toxicity under oxic and hypoxic conditions with any of the compounds studied, survival data obtained under both conditions were used. The calculated incubation times (t) for each drug concentration (c), were fitted to the equation $c = 1/kt^n$ where n and k are constants [16, 17], using linear least squares on the logarithmic transform, as implemented in the RS/1 program on a Microvax 2 (BBN Research Systems). The extracellular concentrations (see below) required to give a surviving fraction of 0.5 in 1 hour were calculated and are tabulated in Table 2, ranked in order of relative uptake (see below), with the appropriate standard errors. Hall *et al.* [16, 17] and Hodgkiss and Middleton [18] showed that, for simpler nitroaryl compounds, the concentration needed for a constant effect was approximately inversely proportional to the square of the exposure time t (i.e. $n \simeq 0.5$). It may be relevant that the time-dependence coefficient n with these nitronaphthalimides seems to fall into two groups, those with $n \simeq 0.3$ and those with $n \simeq 0.7$ (Table 2).

The time course of uptake of some nitronaphthalimides by cells incubated under oxic conditions is shown in Fig. 2. Identical uptake of DM112, one of the more water-soluble compounds, was found in the presence and absence of DMSO. The plateau levels of cellular drug content, reached after 4 hours' incubation with two extracellular drug concentrations have also been summarised in Table 2.

DISCUSSION

Nitronaphthalimides, such as mitonafide, are cytotoxic in *in vitro* test systems [1–3, 5]. Intercalation of naphthalimides to DNA has been proposed as one of the primary causes of toxicity [1, 2, 8] and inhibition of DNA synthesis in a permeabilised cell system suggested that mitonafide acted directly without requiring metabolic conversion [6]. Numerous nitroaromatic compounds have been shown to be selectively toxic to hypoxic cells, e.g. [18, 19] because oxygen interferes with bio-reductive metabolism of the nitro-substituent [20]. We had hoped that in 3-nitronaphthalimides of lower toxicity than mitonafide, toxicity due to intercalation would be less important than toxicity due to reduction of the nitro-group to the amine and more reactive intermediates, thus conferring hypoxia-dependent toxicity. We have previously shown hypoxic-specific metabolism of nitronaphthalimides such as DM113 to yield a range of metabolites including the amine, DM118 [12]. Table 2 shows DM118 to be about 11-fold less toxic than its parent nitro-compound DM113 although uptake is only 2–3-fold less; similarly the amino derivative of mitonafide (NSC 308847) induces less

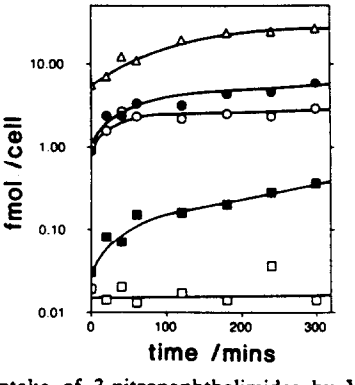


Fig. 2. Uptake of 3-nitronaphthalimides by V79 379A Chinese hamster cells: (●), DM112; (○), DM113; (■), DM124; (△), DM155; (□), DM161. The initial extracellular drug concentration was 0.1 mmol dm^{-3} for each compound.

DNA damage than mitonafide [10]. However, despite this, there was no significant difference in toxicity under oxic and hypoxic conditions for DM113 or any of the other nitro-naphthalimides. This suggests, as with mitonafide [7] that reductive metabolism is relatively unimportant in contributing to toxicity compared with other factors such as intercalation into DNA. It is also possible that toxicity from oxygen radicals formed in the futile redox cycle with the first one-electron reduction product contributes to the oxic toxicity of nitro-naphthalimides [10].

All of the compounds used in this study gave convex survival curves (e.g. Fig. 1b, 1d), with the exception of DM113 and mitonafide which gave concave survival curves (e.g. Fig. 1a, 1c). The shapes of survival curves obtained with DM113 and mitonafide do not reflect depletion of the extracellular drug, and concentrations of DM113 were used which were comparable with those used for other compounds which gave convex survival curves, e.g. DM155 (Fig. 1d). Thus it seems likely that DM113, and possibly mitonafide, have different mechanisms of toxicity than the other compounds.

Uptake of naphthalimides into cells is initially rapid, and reaches a plateau after a few hours (Fig. 2). In some cases, final concentrations inside the cells, summarised in Table 2, may exceed the external concentrations of drugs by as much as two orders of magnitude, presumably reflecting the affinity of the drugs for binding sites such as nucleic acids within cells. A 10-fold change in extracellular concentrations altered uptake into cells by factors of from 1.8 to 26. Ideally, one would wish to measure intracellular uptake at the same concentrations used for cytotoxicity measurements; unfortunately sensitivity considerations preclude this with e.g. mitonafide. The relatively small increase in intracellular concentration of DM163 obtained on a tenfold increase in extracellular concentration, coupled with the highest amount of uptake, presumably reflects a very high affinity of the drug for its binding site which is nearly saturated even with low extracellular concentrations. The uptake data in Table 2 make no allowance for depletion of the drugs in the growth medium; however, uptake studies were carried out under oxic conditions where little metabolic conversion of the compounds occurs, and only DM155 and DM163 were taken up into cells to a great enough extent that their extracellular concentration was significantly reduced. Uptake values given for DM154 are probably too low as our extraction procedure failed to remove all of the brightly coloured drug from cell pellets. The poor penetration of DM161 into cells, and its consequent lack of toxicity, is probably due to an ionised acidic-function (cf. uptake of the carboxylic acid derivative, Ro 31-0258, in this same cell line [21]).

The data in Table 2 show that for these compounds there is a very poor correlation between toxicity and uptake. This suggests that structural features related to drug binding can be separated from those causing toxicity; although as discussed above, metabolic activation is probably not important, the electron withdrawing properties of nitro-substituents make a significant contribution to the overall toxicity of these structures. Brana *et al.* [3] showed that toxicity of substituted naphthalimides to HeLa cells was maximised by a terminal nitrogen on a side chain; substitution of this terminal nitrogen or its replacement by a morpholine group reduced toxicity. Similarly we find that toxicity decreases with increasing substitution of the terminal nitrogen in the series DM113 > DM114 > DM115, that those compounds with no terminal nitrogen are of relatively low toxicity and that the terminal morpholine group of DM159 greatly reduces its toxicity. In this context it is surprising that mitonafide with a di-substituted terminal nitrogen on its side chain should be the most toxic compound tested, especially as its uptake into cells is much less efficient than that of many less toxic compounds in this series.

In conclusion, we find a poor correlation between cellular uptake and toxicity for this series of substituted naphthalimides, indicating that toxicity of these structures is largely influenced by their substituents rather than absolute concentration of the naphthalimide ring. Despite evidence for extensive hypoxic metabolism of nitronaphthalimides, there is no evidence for hypoxia-specific toxicity. The rapid uptake and intracellular concentration of some of these naphthalimides would be expected to result in poor penetration through solid tumours where cells are packed at high densities. Thus these compounds would not be expected to be suitable for targeting against hypoxic cells in tumours.

Acknowledgements—We thank Dr Narayanan for providing mitonafide and Drs P. Wardman and J. Parrick for helpful discussion. This work was supported by the Cancer Research Campaign.

REFERENCES

1. M. F. Brana, J. M. Castellano, A. Jimenez, A. Llombart, F. P. Rabadan, M. Roldan, C. Roldan, A. Santos and D. Vazquez, *Proc. 10th Int. Congress of Chemotherapy* **2**, 1216 (1977).
2. M. F. Brana, J. M. Castellano, C. M. Roldan, A. Santos, D. Vazquez and A. Jimenez, *Cancer Chemother. Pharmac.* **4**, 61 (1980).
3. M. F. Brana, A. M. Sanz, J. M. Castellano, C. M. Roldan and C. Roldan, *Eur. J. Med. Chem.* **16**, 207 (1981).
4. A. Garcia-Gancedo, C. Gil, C. M. Roldan, S. Perez and P. Vilas, *Chemotherapy* **25**, 83 (1979).
5. E. Gonzalez, M. R. Martinez-Larranaga, S. Barrigon, P. Rivera, A. Perez and P. D. Garcia de Jalon, *Proc. 1st Congr. Eur. Biopharm. Pharmacocinet.* **2**, 117 (1981).
6. A. Nishio and E. M. Uyeki, *J. natn. Cancer Inst.* **70**, 1097 (1983).
7. K. D. Paull, M. Nasr and V. L. Narayanan, *Arzneim.-Forsch/Drug Res.* **34**, 1243 (1984).
8. M. J. Waring, A. Gonzalez, A. Jimenez and D. Vazquez, *Nucleic Acids Res.* **7**, 217 (1979).
9. E. C. McCoy, E. J. Rosenkranz, L. A. Petrullo, H. S. Rosenkranz and R. Mermelstein, *Environ. Mutagen.* **3**, 499 (1981).
10. B. K. Sinha, J. Strong, N. W. Gibson and B. Kalyanaraman, *Biochem. Pharmac.* **34**, 3845 (1985).
11. P. Wardman, E. D. Clarke, R. J. Hodgkiss, R. W. Middleton, J. Parrick and M. R. L. Stratford, *Int. J. Radiat. Biol. Phys.* **10**, 1347 (1984).

12. M. R. L. Stratford, E. D. Clarke, R. J. Hodgkiss, R. W. Middleton and P. Wardman, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1353 (1984).
13. A. C. Begg, R. J. Hodgkiss, N. J. McNally, R. W. Middleton, M. R. L. Stratford and N. H. A. Terry, *Br. J. Radiol.* **58**, 645 (1985).
14. R. W. Middleton and J. Parrick, *J. Heterocyclic Chem.* in press.
15. R. W. Middleton, J. Parrick, E. D. Clarke and P. Wardman, *J. Heterocyclic Chem.* **23**, 849 (1987).
16. E. J. Hall, R. Miller, M. Astor and F. Rini, *Br. J. Cancer* **37**, Suppl. 3, 120 (1978).
17. E. J. Hall, M. Astor, R. Osmak, P. Shapiro and L. August, *Int. J. Radiat. Oncol. Biol. Phys.* **5**, 1781 (1979).
18. R. J. Hodgkiss and R. W. Middleton, *Biochem. Pharmac.* **34**, 2175 (1985).
19. J. K. Mohindra and A. M. Rauth, *Cancer Res.* **36**, 390 (1976).
20. R. P. Mason in *Free Radicals and Biology*, Vol. 5. (Ed. W. A. Price), p. 161. Academic Press, New York (1982).
21. M. F. Dennis, M. R. L. Stratford, P. Wardman and M. E. Watts, *Int. J. Radiat. Biol.* **47**, 629 (1985).