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FLUORESCENT MARKERS FOR HYPOXIC CELLS

A STUDY OF NOVEL HETEROCYCLIC COMPOUNDS THAT UNDERGO BIO-REDUCTIVE BINDING

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Abstract—The bioreductive metabolism and binding of nitroaromatic compounds has been suggested as a method for the identification of hypoxic tumour cells. Bound metabolites of suitable nitroaryl compounds (and some other reducible aromatic compounds) may fluoresce, offering an alternative to radiolabelling or NMR etc. as a diagnostic method. In this paper, the synthesis of some heteroaromatic nitro-compounds is given together with the results obtained from testing of these and other mainly nitroaromatic compounds *in vitro* as potential bioreductive fluorescent probes for hypoxic cells in tumours. Compounds were incubated with oxygenated or hypoxic mammalian cell suspensions for various times before evaluation of the cellular fluorescence from bioreductive metabolites by fluorescence microscopy and flow cytometry. Among those compounds yielding fluorescent metabolites in cells, considerable variation in hypoxic:oxic differential fluorescence was observed. The *in vitro* mammalian cell test system showed several of the compounds to be sufficiently promising to merit further investigation *in vivo*.

It is known that hypoxic cells, which are approximately three times less sensitive to radiation than oxic cells, exist in human tumours [1, 2], and the trials with hyperbaric oxygen suggest that they may be important in clinical radiotherapy [3]. It would therefore be of clinical importance to know which tumours contain significant numbers of hypoxic cells, as selected patients could benefit from additional treatments with, for example, hypoxic cell radiosensitizers such as etanidazole or pimonidazole, hyperbaric or normobaric oxygen or high LET radiation.

A number of approaches to the determination of tumour hypoxia all rely on the oxygen-specific inhibition of the reduction of the nitro group, by re-oxidation of the nitro radical anion, the one electron reduction product, by oxygen [4, 5]. Several workers have used isotopically labelled nitroimidazoles such as ^{14}C-labelled misonidazole, which are covalently bound to macromolecules, to visualize areas of hypoxia using autoradiography (e.g. Refs 6–10). Using a fluorine substituted analogue of misonidazole synthesized to allow nuclear magnetic resonance detection of hypoxic areas in tumours [11], Raleigh *et al.* [12] have developed monoclonal antibodies to bound adducts, while *in situ* visualization with γ-ray and positron emission tomography has been tried by a number of workers using halogenated derivatives of misonidazole (e.g. Ref. 13).

An alternative approach is to use the metabolic binding of fluorescent nitroaromatic compounds such as some nitrofurans (e.g. Refs 13–16). The advantage of this last approach is that not only can the fluorescence be visualized in histological sections, but it lends itself to quantitation (including cell population distributions) using flow cytometry with fluorescence-activated cell sorting. We have modified this approach to exploit the quenching of fluorescence by the nitro group, to produce very weakly fluorescent compounds [17]. When these compounds are used as probes for hypoxic cells, it is expected that bioreduction of the nitro group in hypoxic cells will lead to production of fluorescent metabolites [18]. This then gives a differential fluorescence based on both the enzymatic reduction and binding in hypoxia, but also on the quenching of the fluorescence in the un-reduced parent compound.

We have already described two nitroheterocyclic probes for hypoxic cells, a nitroacridine (Nitroakridin 3582) and a nitronaphthalimide [18–21], both of which showed a large hypoxic/oxic differential *in vitro*, which could be measured by either HPLC or flow cytometry. We present here data on a series of nitro-aromatic compounds of varying physico-chemical properties.

MATERIALS AND METHODS

DMSO and other reagents were BDH AnalaR grade. Special gases (air + 5% CO_2, nitrogen + 5% CO_2) were obtained from British Oxygen Co. Infrared spectra were recorded as KBr discs on a Pye Unicam SP 200 spectrometer. ^{1}H NMR spectra were recorded on either a Varian T-60 or CFT-20 spectrometer with tetramethylsilane as internal standard, and mass spectra were obtained on an A.E.I. MS 902 spectrometer. Melting points were

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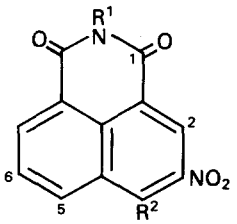
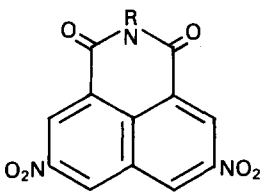
determined in open capillary tubes and were uncorrected.

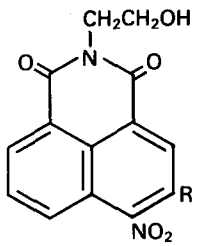
Published methods were used to obtain compounds **9, 12–16** [22], **1** [23], **25** [24], **26** [25], **31** [26], **32** [27], **34** [28], **37** and **38** [29]. Compounds **23** and **42** were generous gifts from Dr Peggy Olive and Dr E. L. Engelhardt, Merck, respectively. Compounds **17, 19, 29, 30, 36** and **39** were obtained from the Aldrich Chemical Co.; **43** and **44** from BDH; **18** and **20–22** from the Sigma Chemical Co.; and **24** from Lancaster Synthesis. The remaining compounds (Fig. 1) were obtained as described below.



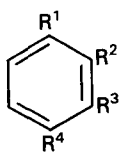
1. $R^1 = CH_2CH_2OH, R^2 = H$
2. $R^1 = CH_2CH(OH)CH_2OH, R^2 = H$
3. $R^1 = (CH_2)_2N$⟨piperazine⟩$NH, 2HCl, R^2 = H$
4. $R^1 = CH_2CH(OH)CH_2NH_2, HCl, R^2 = H$
5. $R^1 = (CH_2)_2NHCH_2CH_2OH, HCl, R^2 = H$
6. $R^1 = (CH_2)_2N(CH_2CH_2OH)_2, HCl, R^2 = H$
7. $R^1 = (CH_2)_3SO_3^-Na^+, R^2 = H$
8. $R^1 = (CH_2)_3NH(CH_2)_3NH_2, 2HCl, R^2 = H$
9. $R^1 = (CH_2)_3N$⟨morpholine⟩$O, R^2 = NHR^1$
10. $R^1 = CH_2CO_2H, R^2 = H$
11. $R^1 = (CH_2)_3N$⟨morpholine⟩$O, R^2 = S(O)Bu^n$



12. $R = CH_2CH_2OH$
13. $R = (CH_2)_3N(CH_2CH_2OH)_2$

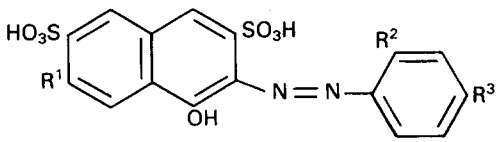


14. $R = H$
15. $R = OH$
16. $R = OMe$

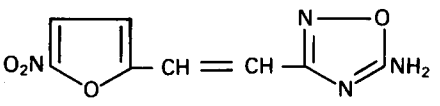


17. $R^1 = SO_2$⟨thiazole⟩$NH_2, R^2 = R^3 = H, R^4 = NO_2$
18. $R^1 = CO_2H, R^2 = NO_2, R^3 = R^4 = H$
19. $R^1 = CH_2CH(NH_2)CO_2H, R^2 = H, R^3 = NO_2, R^4 = OH$
20. $R^1 = NEt_2, R^2 = R^3 = H, R^4 = CPh =$⟨cyclohexadienylidene⟩$^+NEt_2$

Fig. 1. The structures of compounds used in this work.



21. $R^1 = NHCOMe, R^2 = H, R^3 = SO_2NH_2$
22. $R^1 = H, R^2 = R^3 = NO_2$



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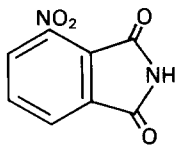

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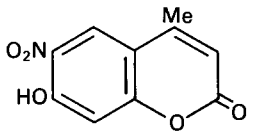

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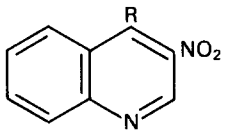
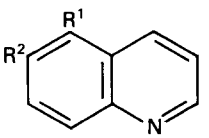
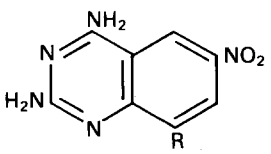


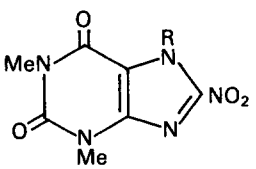
26. R = OH
27. $R = NH(CH_2)_2NEt_2, HCl$
28. $R = NH(CH_2)_3N(CH_2CH_2OH)_2$



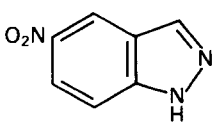
29. $R^1 = NO_2, R^2 = H$
30. $R^1 = NH_2, R^2 = NO_2$



31. R = H
32. $R = NO_2$
33. $R = H, EtSO_3H$

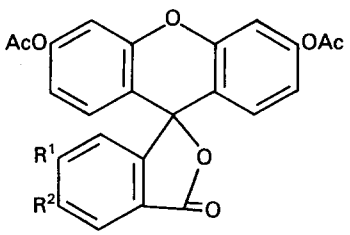


34. $R = CH_3$
35. $R = CH_2CO_2Et$

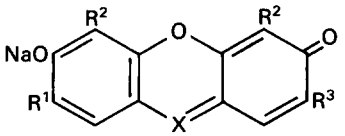


36.

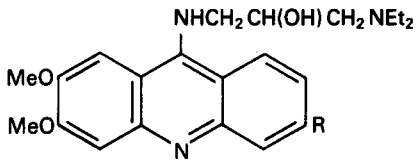
Fig. 1 (continued).



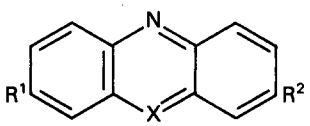
37. $R^1 = H, R^2 = NO_2$
38. $R^1 = NO_2, R^2 = H$



39. $X = C\text{-}C_6H_4CO_2Na\text{-}4, R^1 = R^3 = NO_2, R^2 = Br$
40. $X = N^+ - O^-, R^1 = R^2 = R^3 = H$



41. $R = NH_2, 2HCl, 2H_2O$
42. $R = NO_2$



43. $X = N^+ - Ph, R^1 = NEt_2, R^2 = N = NC_6H_4NMe_2\text{-}4, Cl^-$
44. $X = S^+, R^1 = R^2 = NMe_2, Cl^-$

Fig. 1 (continued).

Synthesis

3-Nitronaphthalimides, **2–6, 8, 10** *and* **11***: general procedure*. These compounds were prepared by the reaction of the corresponding naphthalic anhydride with the appropriate aliphatic amine. In a typical experiment the amine (10 mmol) in absolute ethanol (25 mL) was added dropwise to a solution of the anhydride (10 mmol) also in ethanol (50 mL). The reaction mixture was then stirred for 24 hr at room temperature and the solid product obtained either by filtration or by evaporation of the solvent under reduced pressure. The yield, solvent for crystallization, m.p. and elemental analysis data for these compounds are given in Table 1. The NMR and i.r. spectral data for the compounds were consistent with those obtained for similar compounds [23].

N-*(3-Propylsulphonic acid)-3-nitronaphthalimide sodium salt*, **7**. *N*-Nitronaphthalic anhydride (2.0 g, 8.23 mmol), sodium 3-aminopropylsulphonate (1.63 g, 8.27 mmol) and ethanol (40 mL) were heated at reflux for 2 hr. Boiling water was added to the resulting suspension until solution occurred. The reaction mixture was filtered while hot and then cooled to 5°. The crude product was filtered off and crystallized from a mixture of ethanol and aqueous sodium chloride [1% (w/v) solution in water] to give the *title compound* (1.8 g, 61%), m.p. >300°: υ_{max} 3450, 1700, 1660, 1600, 1340, 1200, 1050, 780 and 760 cm^{-1}, $\delta(d_6\text{-DMSO})$ 2.10 (2H, quintet, *J* 7 Hz, $CH_2CH_2CH_2$), 2.50 (2H, t, *J* 7 Hz, CH_2SO_3), 4.10 (2H, t, *J* 7 Hz, naphth-CH_2), 8.00 (1H, t, *J* 9 Hz, 6-H), 8.50–8.83 (2H, m, 5- and 7-H), 8.85 (1H, d, *J* 2 Hz, 4-H) and 9.43 (1H, d, *J* 2 Hz, 2-H); (Found: C, 46.33; H, 3.25; N, 6.87; $C_{15}H_{11}N_2SO_7Na$ requires C, 46.63, H, 2.85; N, 7.25%.

4-*(2-(Diethylamino)ethylamino)-3-nitroquinoline hydrochloride*, **27**. 4-Chloro-3-nitroquinoline (900 mg, 4.3 mmol), 2-diethylaminoethylamine (500 mg, 4.3 mmol) and propan-2-ol (12 mL) were heated at reflux for 50 min. The reaction mixture was left overnight and the resulting yellow precipitate filtered off at the pump to afford the pure *title compound* (780 mg, 52%), m.p. 152–153°; υ_{max} 3270, 2980, 2440, 1640, 1590, 1540, 1350, 1170, 815 and 780 cm^{-1}; $\delta(d_6\text{-DMSO})$ 1.23 (6H, t, *J* 7 Hz, 2 × CH_3), 3.14 (4H, quartet, *J* 7 Hz, 2 × NCH_2CH_3), 3.53 (2H, t, *J* 6 Hz, NCH_2CH_2), 3.79 (2H, broad s, exchanged with D_2O, NH_2) 4.02 (2H, t, *J* 6 Hz, NCH_2CH_2), 7.58–7.90 (3H, m, 5. 6- and 7-H) 8.35 (1H, d, *J* 8 Hz, 8-H) and 9.03 (1H, s, 2-H): (Found: C, 54.56; H, 6.10; N, 16.39; $C_{15}H_{21}ClN_4O_2$ requires C, 55.46; H, 6.51; N, 17.25%).

4-*(3-Bis{2-hydroxyethyl}propylamino)-3-nitroquinoline*, **28**. 4-Chloro-3-nitroquinoline (4.3 mmol) was allowed to stand with *N,N*-bis(2-hydroxyethyl)-propane 1,3-diamine (4.3 mmol) in propan-2-ol (15 mL) at room temperature for 3 hr. The solid was filtered off and crystallized from ethanol to yield the product (71%), m.p. 172–173° (Found: C, 51.8; H, 6.17; N, 14.98. $C_{16}H_{23}ClNO_4$ requires C, 51.82; H, 6.24; N, 15.11%).

2,4-*Diamino-6-nitroquinazoline ethanesulphonate*, **33**. The free base, in warm ethanol, was treated with

Table 1. 3-Nitronaphthalimides

Compound	Yield (%)	Solvent	m.p. (°C)	Formula	Found (%)			Calculated (%)		
					C	H	N	C	H	N
2	70	aq. EtOH	186–187	$C_{15}H_{12}N_2O_6$	56.76	4.06	9.10	56.96	3.80	8.86
3	58	aq. HCl-EtOH	200 (dec.)	$C_{18}H_{20}Cl_3N_4O_2$	50.71	4.74	13.12	50.59	4.68	13.11
4	40	aq. HCl-EtOH	248–150	$C_{15}H_{14}ClN_3O_5$	51.01	3.96	11.94	51.21	3.98	11.95
5	74	aq. HCl-EtOH	286–287	$C_{16}H_{16}ClN_3O_5$	52.56	4.40	11.61	52.53	4.38	11.49
6	60	aq. HCl-EtOH	193–195	$C_{19}H_{22}ClN_3O_6$	53.93	5.18	9.93	53.84	5.19	9.92
8	66	aq. HCl-EtOH	274–276	$C_{18}H_{22}Cl_2N_4O_4$	49.94	5.40	12.71	50.35	5.13	13.03
10	53	aq. EtOH	236–238	$C_{14}H_8N_2O_6$	55.43	3.10	9.10	56.01	2.69	9.33
11	62	EtOH	121–122	$C_{23}H_{28}N_2O_4S$	64.66	6.76	6.74	64.46	6.59	6.54

ethanesulphonic acid. On cooling the solution, the salt crystallized out in near quantitative yield, m.p. >300° (Found: C, 38.36; H, 4.04; N, 21.98. $C_{10}H_{13}N_5O_5S$ requires C, 38.10; H, 4.13; N, 22.22%).

7-Ethoxycarbonylmethyl-8-nitrotheophylline, **35**. 8-Nitrotheophylline (0.45 g, 2 mmol) was converted into its dry sodium salt and dissolved in dry DMF (20 mL). Ethyl chloroacetate (0.3 g, 2.4 mmol) was added and the mixture heated to 140° in the presence of sodium iodide (a few small crystals) for 3 hr. The mixture was poured into water and the product isolated by extraction with chloroform and then chromatographed on silica gel with ethyl acetate as eluant to give an oil (0.275 g, 44%). The oil was distilled, b.p. 250° at 0.5 mm Hg, to give the ester as a viscous oil which solidified, m.p. 42–43° (Found: C, 42.06; H, 4.21; N, 22.7. $C_{11}H_{13}N_5O_6$ requires: C, 42.44; H, 4.18; N, 22.5%).

3 - Amino - 6,7-dimethoxy-9(2-hydroxy-3-diethylamino)propylaminoacridine, **41**. The corresponding nitro compound, **42**, was reduced with hydrogen in the presence of palladium charcoal in ethanol. The reduction proceeded smoothly at room temperature. Removal of the catalyst and evaporation of the solvent gave the crude product which was crystallized from dilute hydrochloric acid to afford the amine dihydrochloride dihydrate, m.p. 238–240° (Found: C, 52.12; H, 6.95; N, 10.87. $C_{22}H_{36}Cl_2N_4O_5$ requires C, 52.07; H, 7.10; N, 11.04%).

Biology

V79 379A Chinese hamster cells were maintained as exponentially growing suspension cultures in Eagle's Minimal Essential Medium with Earle's salts, modified for suspension culture, with 7.5% foetal calf serum. The method of incubating cells with drugs for measuring the production of fluorescent products has been described [20, 30]. Because of the poor water solubility of some of the compounds used in this work, many of the drugs were initially dissolved at 10–20 mmol/dm^{-3} in dimethylsulphoxide (DMSO) and small volumes added to cell suspensions to give the appropriate drug concentration. The final concentration of DMSO was 1% (v/v) or less.

Samples from hypoxic and aerobic cell suspensions incubated with drugs for various times were initially evaluated by fluorescence microscopy using excitation with UV, UV + violet or blue excitation wavelengths (Table 2). Further evaluation by flow cytometry was then carried out on promising compounds, using the fluorescence microscopy observations as a guide.

Flow-cytometry analysis of cells for fluorescent products was carried out in an Ortho Systems 50 cytofluorograph using a Coherent 5W laser. For most compounds the optimum excitation wavelength was at 458 nm and green fluorescent emission was collected at 90° to the incident beam between 510 and 560 nm (Table 2). In some cases where optimum excitation was at 365 nm, the total fluorescent emission from the drug metabolites was collected at 90° to the incident beam and separated from the scattered excitation light by a cut-off filter that passed all wavelengths above 390 or 410 nm (Table

Table 2.

	Fluorescence microscopy				Flow cytometry		
	Microscope filter set	Intensity hypoxic fluorescence	Intensity oxic fluorescence	*t* max. /hr	Intensity hypoxic fluorescence	Intensity oxic fluorescence	*t* max. /hr
1	c	+	+	5.0	70	4	1.0
2	c	+	+	5.0			
3	c	+++	++	2.5	190	5	1.0
4	c	+++	+	2.0	130	1	2.0
5	b	+++	+	0.5	51	2	1.0
6					95	9	0.5
7	c	−	−	4.0			
8	abc	++	+	4.0			
9	abc	+	+	4.0			
10	abc	−	−	2.0	nf	nf	2.0
11	b	+++	+++	1.0			
12	abc	−	−	2.0			
13	abc	−	−	2.0			
14	c	+++	+++	4.0	380	380	4.0
15	abc	−	−	4.0			
16	b	+++	+++	0.5	18,000	18,000	0.5
17	abc	−	−	4.0			
18	a	+	+	5.0			
19	abc	−	−	4.0			
20	abc	−	−	5.0			
21	abc	−	−	5.0			
22	abc	−	−	5.0			
23	b	+	−	2.0	52	6	4.0
24	abc	−	−	5.0			
25	abc	−	−	5.0			
26	abc	−	−	5.0			
27	abc	−	−	5.0			
28	abc				240	140	6.0
29	abc	−	−	4.0	nf	nf	4.0
30	a	+	−	2.0			
31	a	+	−	0.5	370	240	0.5
32	a	+	−	4.0			
33	abc	−	−	5.0			
34	abc	−	−	5.0			
35	abc	+	−	5.0	nf	nf	5.0
36	abc	−	−	4.0			
37	c	+++	+++	1.0			
38	c	+++	+++	1.0			
39	c	++	+	5.0	320	100	4.0
40					3300	200	1.0
41	c	+++	+++	0.5			
42	c	+++	+	2.0	320	2	2.0
43	c	+	−	5.0			
44	a	+	+	5.0			

For fluorescence microscopy, drug concentrations were all 10^{-4}M except for compounds **1, 30** and **39** which were used at 5×10^{-5}, 8×10^{-5} and 5×10^{-4} M, respectively. Band-pass excitation filters were (a) 340–380 nm (b) 355–425 nm and (c) 450–490 nm, matched with long-pass emission filters (a) >430 nm, (b) >460 nm and (c) >515 nm. Fluorescence was excited at 458 nm in the flow cytometer and collected in the green channel, except for compounds **16, 28, 29, 31** and **40** where excitation was at 366 nm and all wavelengths >390 nm were collected. All drug concentrations were 10^{-4} M except for compounds **23, 39** and **42** where concentrations of 2×10^{-5}, 5×10^{-4} and 10^{-5} M, respectively, were used. Mean fluorescence intensities (in arbitrary units) measured by flow cytometry have been normalized for constant PMT gain and constant laser excitation power of 100 mW.

2). Forward-scattered light at the excitation wavelength was also collected and used to discriminate non-cellular debris (low scatter). The instrument was calibrated by measuring the mean fluorescence from cells, incubated with Nitroakridin 3582, **42**, or the 3-nitronaphthalimide, **4**, for 2 hr, while varying the photomultiplier gain and laser excitation energy.

RESULTS

The structures of the compounds in this work are given in Fig. 1. The results of their evaluation in the mammalian cell test system by fluorescence microscopy and flow cytometry are shown in Table 2. The cellular fluorescence detected by the flow cytometer was approximately exponentially

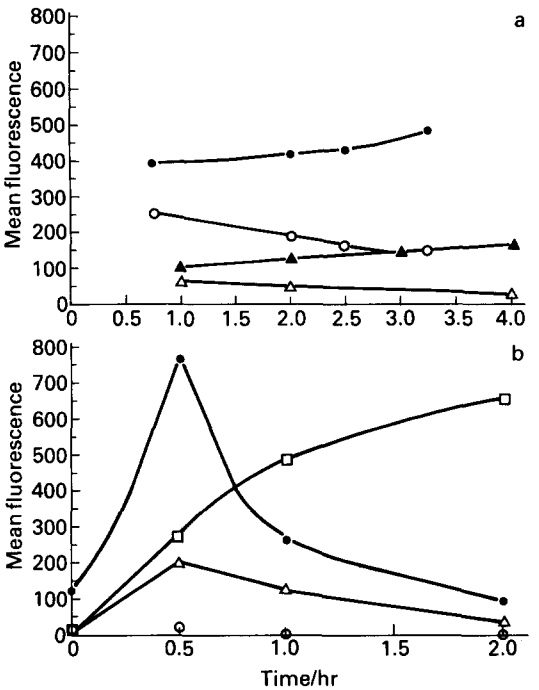


Fig. 2. Typical time-courses of cellular metabolism of nitroaromatic compounds to fluorescent derivatives: (a) **23** incubated with (▲) hypoxic cells or (△) oxic cells; **39** incubated with (●) hypoxic cells or (○) oxic cells. (b) **3** incubated with (●) hypoxic cells or (○) oxic cells; **6** incubated with hypoxic cells (△); **42** incubated with hypoxic cells (□). Data for oxic cells incubated with **6** or **42** is similar to that for oxic cells incubated with **3** and has been omitted for clarity.

dependent on the photomultiplier gain setting but was much less dependent on changes in the output excitation power of the laser beam. When some of the compounds were incubated with aerobic or hypoxic Chinese hamster cells, the amount of fluorescence varied, under both conditions, in a time-dependent manner (Fig. 2) which was characteristic of each compound. Typically, the amount of cellular fluorescence increased to a maximum and then diminished with further incubation. It was therefore important that each compound was screened over a range of incubation times, as use of a single standard time might have prevented differential fluorescence (hypoxic:oxic) from being observed.

DISCUSSION

Fluorescence microscopy was a good method of detecting cellular fluorescence since it has at least as great a sensitivity as flow cytometry; this technique was used as an initial screen for the selection of compounds suitable for further evaluation. In some cases, faint fluorescence observed by fluorescence microscopy could not be detected in the flow cytometer. This may be due partly to the restricted wavelengths available from the argon ion laser used as an excitation source in the flow cytometer [18]. In particular, no laser wavelengths were available between 366 and 458 nm; this region was covered by the excitation filters on the fluorescence microscope, and some compounds appeared to produce metabolites with a cellular fluorescence best observed when excited in this wavelength band. Many of the compounds evaluated as potential fluorescent probes for hypoxic cells failed to produce any cellular fluorescence in the *in vitro* test system. This could reflect low penetration of the cells by some of the parent compounds, inadequate metabolism to fluorescent derivatives or production of non-fluorescent metabolites. In addition, fluorescent metabolites will only reach detectable levels within cells if they can bind covalently to cellular macromolecules or concentrate within cells by some other means such as intercalation with DNA.

However, microscopy was not a good method for judging the extent of the hypoxic:oxic differential fluorescence, although it was possible to make an estimate and this is indicated in Table 2. Flow cytometry was found to be the better method for measuring the differential in fluorescence but the limitations in excitation wavelengths available may have prevented the maximum cellular fluorescence and differential fluorescence from being observed with some compounds. We have previously reported that several cellular metabolites, with different fluorescent properties and different oxygen dependence of production, were found from one of the nitronaphthalimides (**4**) [21].

N-Alkyl naphthalimides have very little solubility in water and, in the main, the alkyl chains were designed to produce increased water solubility through the incorporation of either hydroxyl or amino substituents, or the presence of both groups. The compounds having basic substituents, e.g. **3–6**, generally showed more intense fluorescence in hypoxic cells than the non-basic compounds, **1** and **2**, and also showed a better differential effect between hypoxic and oxic cells (Table 2). However, neither the basic **13** nor the non–basic **12** dinitro compounds showed any fluorescence in hypoxic cells. A possible explanation for the lack of fluorescence in these two cases is that metabolic reduction of only one nitro group occurred. We have previously shown that uptake of the acid **10** into cells is very poor [30]; low uptake because of an ionized acidic function is almost certainly the explanation for the lack of fluorescence with **7** and **10**.

In contrast to some of the results obtained with the 3-nitronaphthalimides, both of the 4-nitro analogues **14** and **16** showed strong and very similar fluorescence in both hypoxic and oxic cells. The displacement of a nitro group from the 4-position of the naphthalimide nucleus has been shown to occur readily in the presence of thiolate ions and it seems likely that this process has occurred in both oxic and hypoxic cells [22]. The observation that the fluorescence intensities measured on the flow cytometer for each compound in the two extreme oxygen tensions are very similar may mean that the displacement reaction with (e.g. glutathione) has occurred much more readily than the reduction process in the hypoxic cells, and may well have been catalysed by cellular glutathione-*S*-transferases.

The acidic *o*-nitrophenol derivative **15** behaved differently, presumably due to ionization of the hydroxyl group with concomitant decreased susceptibility of the nitro group to be displaced and increased difficulty for the ionized species to penetrate the cell membrane. It is possible that the 3-nitronaphthalimide, **11**, showed fluorescence in both oxic and hypoxic cells due to electron withdrawal of the sulphoxide group and consequent replacement of the 3-nitro group by thiols, but this is conjecture.

The naphthalimides suffer from the potential disadvantage of being intercalators and therefore less available for transport to a tumour *in vivo*. Our attention therefore turned to less complex aromatic nuclei with the potential to produce fluorescence. Some very simple benzene derivatives give high fluorescence, e.g. anthranilic acid derivatives which are produced when isatoic acid is used as a fluorescence labelling agent. However, the four simple benzene derivatives we investigated, whether acidic, as in **18**; basic, as for **17** and **20**; or almost neutral **19**, gave disappointing results: only **18** showed any fluorescence *in vitro* and then fluorescence was shown in both oxic and hypoxic cells. The naphthalene acids, **21** and **22**, probably did not get into cells and therefore gave no cellular fluorescence.

At this stage, we tested our assay system with one of the first reported fluorescent probes for hypoxia, *trans*-5-amino-3-[(5-nitro-2-furyl)vinyl]-1,2,4-oxadiazole (NFVO), [14, 15]. As expected NFVO, **23**, showed a differential fluorescence effect, both through the microscope and with the flow cytometer, although neither the fluorescence intensity nor the differential effect were as marked as with some of the basic naphthalimides. (e.g. **4** and **5**). Since a differential effect was shown by NFVO it is reasonable to assume that neither a Michael type of addition of thiol to the double bond of NFVO nor nitro group displacement by thiol occurred to any significant extent.

Two bicyclic compounds which could be considered to be simple benzene derivatives, e.g. 3-nitrophthalimide **24**, and the coumarin **25**, both gave no fluorescence with oxic or hypoxic cells. In view of the observation that basic substituents in the naphthalimides were advantageous, we then turned our attention to basic heterocyclic compounds, but none of the quinoline derivatives, **26–29**, showed any useful effects though **30**, carrying an amino substituent on the carbocyclic ring, did show a weak differential fluorescence under the microscope. Other basic heterocycles investigated were pteridines **31–33**, purines, **34** and **35**, and indazole **36**. Of these, only 6-nitro- and 6,8-dinitro-2,4-diaminopteridine, **31** and **32**, respectively, showed any fluorescence upon incubation and both exhibited some differentiation between oxic and hypoxic cells. In the case of **31** this weak effect was confirmed by flow cytometry. The puzzling results from this group of compounds are those obtained with 8-nitrocaffeine, **34** and the ester, **35**. Nitrocaffeine showed no fluorescence but we know that nitrocaffeine is a good radiosensitizer *in vitro* [22] and produces this effect in part by endogenous thiol depletion through a nitro group displacement reaction. The thiol addition products may not be retained in the cells unlike those from the intercalating naphthalimides.

Studies of linear tricyclic heteroaromatic fluorophores showed that, in the fluorescein series, a nitro group on the phenyl substituent, (e.g. **37** and **38**), did not produce any differential effect; however, the presence of a nitro group on the chromophoric tricyclic system, as in nitro-eosin **39**, did produce a small differential effect. Resazurin **40**, where the reducible group is the *N*-oxide function, showed a bigger fluorescence differential which is significant in that the compound may be used as an indicator of the selective reduction of the *N*-oxide function in hypoxic cells. The nitroacridine **42** showed a marked differential effect, whereas the corresponding amino compound **41** which is its main cellular metabolic [21] showed fluorescence in both oxic and hypoxic cells as expected. Finally, the non-nitro, cationic, tricyclic compounds Janus Green and Methylene Blue, **43** and **44**, respectively, were investigated but only Janus Green showed a differential effect.

The aim of this survey was to explore as wide a range as possible of candidates for fluorescent probes for hypoxic cells. The factors contributing to the range of hypoxic : oxic differential in metabolic nitro-group reduction are complex and not particularly well known except possibly in the case of nitroimidazoles, but even restricting the problems to cell suspensions *in vitro*, probably include redox potential, enzyme substrate specificity, transport across membranes and binding ability to protein or DNA. Several of the compounds evaluated in this work gave rise to fluorescent products when incubated with hypoxic, but not oxic, mammalian cells *in vitro* and appear to be suitable for further evaluation *in vivo* (e.g. **4** and **42**). These structures could provide the basis for the synthesis of further analogues, where the pharmacological factors, for example, which might control their use *in vivo* are also considered.

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