Fluorescent Markers for Hypoxic Cells: A Study of Nitroaromatic Compounds, with Fluorescent Heterocyclic Side Chains, That Undergo Bioreductive Binding

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Several novel compounds having both a 2-nitroimidazole nucleus and a fluorescent ring system in their molecular structure were prepared and evaluated as potential fluorescent probes for hypoxia. Bioreduction of nitroimidazoles, which is inhibited by oxygen, is known to lead to binding of bioreductive metabolites to cellular macromolecules and this provides a mechanism for binding the fluorescent moiety to hypoxic cells. These compounds can incorporate a wide range of fluorophors and can therefore be designed to suit the laser-line wavelengths available for excitation of fluorescence in the flow cytometer. Several nitroimidazoles with naphthalimide side chains were rapidly taken up into cells and became concentrated in the cells, thus reducing their concentration in the extracellular medium. This suggests a potential microscopic bioavailability problem with probes of this type when used in vivo as they would become progressively depleted in the extracellular fluid as they diffused from blood vessels, through layers of packed cells in tumors, to the hypoxic cells where they could undergo hypoxia-specific metabolism. Synthesis of nitroimidazoles with coumarin fluorophors led to several potentially useful probes for hypoxia; substituents on the coumarin fluorophor had a marked effect on the cellular fluorescence of these compounds.

The existence of poorly oxygenated radioresistant cells in tumors is thought to be one of the factors contributing to local failure of radiotherapy and tumor regrowth.[1,2] A simple clinical test for these hypoxic cells in tumors could enable radiotherapy to be optimized for individual patients on the basis of the oxygen status of their tumors. Adjuncts to radiotherapy such as hypoxic cell sensitizers and hyperbaric or normobaric oxygen could be administered to those patients most likely to benefit from them.

Of the various methods that have been proposed for determining the hypoxic cell fraction of tumors, several are based on the hypoxia-dependent bioreductive metabolism of a labeled 2-nitroimidazole, which results in binding of labeled fragments of the original compound to cellular macromolecules. Various labels have been proposed, including ^{3}H, ^{14}C, ^{75}Br, ^{76}Br, ^{77}Br, and ^{19}F.[3-8] Although [^{3}H]misonidazole has been administered to small numbers of patients with treatment-resistant tumors,[9] the need for prolonged autoradiography to detect labeled regions of tumors could be a disadvantage in a routine clinical test. NMR tomography has been used experimentally to detect bioreductively bound metabolites of fluorinated nitroimidazoles and such bound metabolites may be also detected by fluorescence immunohistochemistry.[10,11]

The use of fluorescent nitroaromatic compounds has also been suggested for identifying hypoxic cells (e.g. refs 12-18). The nitro group quenches the fluorescence of the aromatic ring system, but on bioreduction of the nitro group the compound becomes more fluorescent.[16] Thus the hypoxia-specificity of labeling results from both the hypoxia-dependence of nitro-group bioreduction and from the increase in fluorescence from bioreduction. Many of the structures used in model experiments in vitro have been large planar molecules with intercalating properties;[15,16,19] fluorescent metabolites became localized to the nuclear region of cells because of their affinity for DNA.

In this paper, fluorescent labels covalently bound to a 2-nitroimidazole are used to identify hypoxic cells. The side chain of the 2-nitroimidazole misonidazole binds to cellular constituents as efficiently as a ring label.[3] In the fluorescent hypoxic probes described here, the fluorophors are selectively bound to cellular constituents of hypoxic cells as a result of bioreductive metabolism of the nitroimidazole. Data are presented on a group of compounds of this type with a range of different fluorescent moieties.

Chemistry

2-Nitroimidazole (azomycin) was converted into its anion by reaction with sodium methoxide in DMF and alkylated by reaction with *N*-(3-bromopropyl)- (1) and *N*-(5-bromopentyl)-4-aminonaphthalimide (3) to give *N*-(nitroimidazolyl)-4-aminonaphthalimides 5 and 6, respectively (since this work was done we have developed a method for the alkylation of azomycin which is generally more efficient than the one reported here[20]). Interestingly, the 4-acet-

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amidonaphthalimides have been shown[21,22] to have a higher fluorescence quantum yield than the corresponding amino compounds and, since only a low concentration of the fluorescent probe is likely to be present in the hypoxic cells of a tumor in vivo, amino compound **5** was acetylated to obtain acetamido derivative **7**. This compound was also obtained by treatment of acetamido-*N*-(bromopropyl)-naphthalimide (**2**) with the azomycin anion. An attempt was made to obtain a more water-soluble compound by the synthesis of **9**, which contains both amine and hydroxyl groups in a side chain and retains an amide function as the 4-substituent on the naphthalimide nucleus. To this end, **5** was acylated with chloroacetyl chloride to give **8**, and subsequent nucleophilic displacement of the reactive chlorine atom in **8** with *N*,*N*-bis(2-hydroxyethyl)-1,3-diaminopropane gave the desired product, which was isolated as its trihydrochloride **9**.

The possibility of fluorescence quenching by energy or electron transfer between the naphthalimide fluorophor and the electron-affinic nitroimidazole nucleus[23] was not seen to be a problem since fluorescence was not required in the oxic cells where the nitroimidazole moiety was expected to be present. However, the possibility of fluorescence quenching by interaction between the naphthalimide nucleus and the reduction products of the nitroimidazole moiety was considered to be a potential problem even though these reduction products were likely to be much less electron-affinic than the nitroimidazole nucleus. In an attempt to obtain some evidence concerning the possible existence of such an effect in these reduction products, a compound containing a longer alkyl chain separating the two aromatic nuclei was prepared. Alkylation of 4-aminonaphthalimide with 1,9-dibromononane gave **4**, and subsequent reaction of this with azomycin yielded **10**.

Potential fluorescent probes of this general type containing either the quinazoline or coumarin nucleus as the fluorophor were prepared. Quinazoline **12** was obtained from the antihypertensive agent Prazosin (**11**)[24] by the reaction of **11** with 1-(2,3-epoxypropyl)-2-nitroimidazole (**13**).[25] Coumarins have been shown to be effective fluorophors, particularly derivatives of 7-hydroxycoumarin (umbelliferone), and the useful intermediate ethyl 7-methoxycoumarin-3-carboxylate (**14**) was readily prepared in one step.[26] The ester group was found to be readily susceptible to nucleophilic attack. Thus the reaction of **14** with 2-(2-aminoethyl)aminoethanol at room temperature gave amide **15**, and treatment of **15** with epoxide **13** caused attack at the secondary amine function to yield **16**, a potential hypoxia probe.

A series of coumarin derivatives which does not contain the potentially readily biodegradable amide function was prepared. 3-Methyl-6-methoxycoumarin (**17**) and 3-methyl-6,7-dimethoxycoumarin (**18**) were prepared and converted to their respective 3-(bromomethyl) derivatives **19** and **20** by the action of NBS. These bromomethyl derivatives alkylated azomycin anion to give the potential fluorescent probes **21** and **22**. In addition, isomeric 4-substituted coumarins, **23** and **24** were prepared from the commerically available 4-(bromomethyl)-6,7-dimethoxycoumarins. For comparison, 4-nitrobenzoate **25** was prepared. Finally in this series, compound **27**, containing a sulfide, secondary amine, and secondary alcohol group in the chain, was synthesized by reaction of 4-(bromomethyl)-6-methoxycoumarin with 2-aminoethanethiol to give sulfide **26**, and subsequent reaction of this with epoxide **13** afforded **27**.

Table I. Ultraviolet and Fluorescence Spectral Data for the Naphthalimides and a Quinazoline

	UV spectra[a]		fluorescence spectra[a,b]	
entry	λ_{max}, nm	log ϵ	emission λ_{max}, nm	ϕ_f[c]
2	351	4.09	466	1.0
	362	4.12	467	0.94
	364	4.12	468	0.93
5	351	3.40	528	0.01
	364	3.31	530	0.03
	433	4.04	530	0.04
6	351	3.38	529	0.13
	364	3.31	530	0.12
	432	4.02	529	0.10
	458	3.87	528	0.04
7	351	2.16	467	0.11
	357	2.17	467	0.11
	364	2.16	467	0.11
9	351	4.17	458	0.03
	356	4.18	459	0.03
	364	4.16	459	0.03
11	343	3.71	396	0.20
	351	3.67	396	0.17
	364	3.39	399	0.07

[a] Measured for methanolic solutions. [b] For 10^{-7} M solutions. [c] Relative quantum yields (ϕ_f) from two or more measurements.

The results obtained from studies of the ultraviolet and fluorescence spectra of some of the compounds prepared in this investigation are given in Table I. We have already shown[21] that 4-aminonaphthalimides having an alkyl chain on the imide nitrogen show fluorescence with a relative quantum yield ϕ_f of about 0.1 whereas 4-nitronaphthalimides carrying a similar imide substituent show only very weak fluorescence with, typically, $\phi_f < 0.0001$. The presence of the nitroimidazole nucleus at the end of the chain removed from the fluorophor significantly decreased the intensity of the fluorescence, e.g. **5** has $\phi_f = 0.03$.

4-Acetamidonaphthalimides showed much stronger fluorescence than the corresponding amines[21] and this effect has been reinvestigated recently.[22] Not unexpectedly therefore, *N*-(bromoalkyl)acetamidonaphthalimide **2** has $\phi_f = 0.91$. When the fluorescence of mixtures containing **2** and 1-methyl-2-nitroimidazole in molar ratios spanning the range 1:0.1–1:10 were examined, no significant changes in the frequency of the maximum fluorescence or ϕ_f value were observed. The limited evidence available appeared to show that the reduction in fluorescence efficiency observed in **5** was due to an intramolecular effect. Some support for this idea was derived from the observation that **6**, in which the chain was longer than in **5** (where $\phi_f = 0.03$), has a ϕ_f value of 0.01. As expected, 4-acetamido derivative **7**, corresponding to 4-amino compound **5**, showed a greater fluorescence efficiency $\phi_f = 0.11$, but the more complex 4-acetamido derivative **9** had $\phi_f = 0.03$.

The intermediate quinazoline **11** was expected to show similar fluorescence properties to that of the bioreduction product from the candidate fluorescent probe **12**. Quin-

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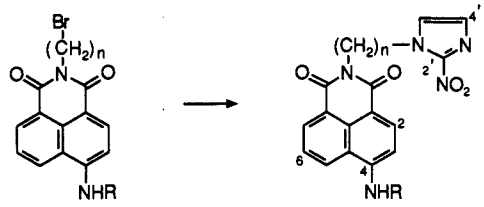
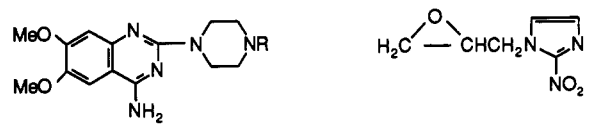
Scheme I. Structures of Compounds 1–13



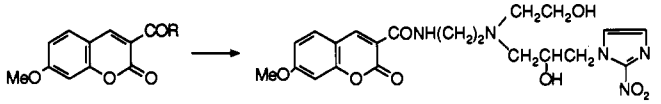
1. R = H, n = 3
2. R = Ac, n = 3
3. R = H, n = 5
4. R = H, n = 9
5. R = H, n = 3
6. R = H, n = 5
7. R = Ac, n = 3
8. R = $COCH_2Cl$, n = 3
9. R = $COCH_2NH(CH_2)_3N(CH_2CH_2OH)_2$, 3HCl, n = 3
10. R = H, n = 9



11. R = H
12. R = $CH_2CH(OH)CH_2$

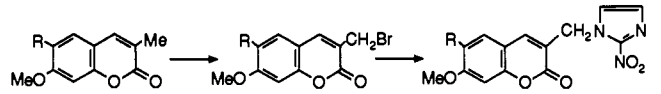
13

Scheme II. Structures of Compounds 14–27

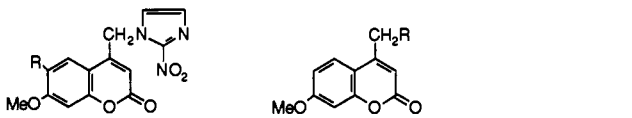


14. R = OEt
15. R = $NH(CH_2)_2NH(CH_2)_2OH$

16



17. R = H
18. R = OMe
19. R = H
20. R = OMe
21. R = H
22. R = OMe



23. R = H
24. R = OMe
25. R = $OCOC_6H_4NO_2$-4
26. R = $SCH_2CH_2NH_3^+Br^-$
27. R = $S(CH_2)_2NHCH_2CH(OH)CH_2$

azoline 11 showed $\phi_f = 0.2$ and a Stokes shift of about 50 nm. This compared with $\phi_f = 0.94$ and 105 nm for **2**. Thus, on purely chemical properties, **7** appeared to have more potential as a hypoxia probe than **12**.

Similar changes in fluorescence efficiency were observed for the coumarins as had been found for the naphthalimides. For example, in the series with 3-substituents, 7-methoxy- (**17**) and 6,7-dimethoxy-3-methylcoumarin (**18**) had ϕ_f of 0.14 and 0.25, respectively, in line with the increased fluorescence efficiency already recognized for the dimethoxy compounds.[27] The corresponding compounds having 3-[(nitroimidazolyl)methyl] substituent (**21** and **22**) have, for our purposes, the advantageously lowered ϕ_f values of 0.016 and 0.062, respectively.

Biological Results and Discussion

The novel compounds shown in Scheme I and II represent a new approach to the design of fluorescent probes for detecting hypoxic cells and consist of a fluorescent group attached, by a side chain, to a 2-nitroimidazole. Previous attempts to synthesise fluorescent probes for hypoxic cells have been based on nitration of fluorescent molecules.[28] There the fluorescence quenching resulting from the addition of a nitro group was seen as an advantage as it eliminated spurious fluorescent signals from residual parent compound within the cells. However, this approach limits the range of nitroaromatic structures to those where the reduced product is likely to have suitable fluorescence properties for excitation at a suitable laser wavelength and there may be conflicting requirements between the biological and fluorescent properties of hypoxic cell probes of this type. In the present class of compounds, the two functions of fluorescence and bioreductive binding are associated with separate parts of the molecule so that each can, in principle, be separately optimized. For example, blue light (458 nm) is a suitable excitation wavelength for the naphthalimide ring system

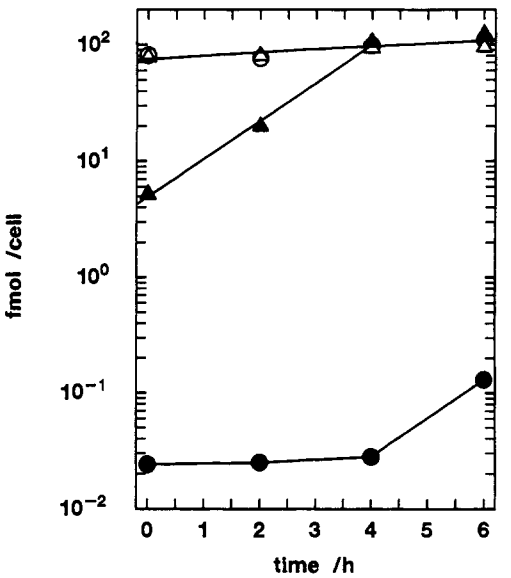


Figure 1. The time course of uptake into aerobic V79 Chinese hamster cells at 37 °C of compounds with a naphthalimide fluorescent label. All extracellular drug concentrations were 10^{-4} M; ca. 0.1 fmol $cell^{-1}$ corresponds to equilibration with the extracellular space. For 10^{-4} M misonidazole, intracellular concentrations of 0.07 mmol dm^{-3} would be expected:[32] (○) **5**; (△) **6**; (●) **7**; (▲) **10**.

5–10, and the greenish fluorescence can be readily observed with a fluorescence microscope or by flow cytometry. If ultraviolet laser wavelengths are available, coumarins are suitable fluorescent labels (**16**, **21**, **22**, and **25–27**) and have shown good discrimination between oxic and hypoxic cells with flow cytometry (Table II). However, the low sensitivity of the eye to the bluish fluorescence from these compounds makes them less suitable for investigations involving microscopy.

Cell-uptake studies showed that some of the nitroimidazoles with naphthalimide sidechains (**5**, **6**, and **10**, Table II, Figure 1) very rapidly accumulated at high concentrations in cells and were bound firmly enough to withstand repeated washing with PBS (although not extraction with ethanol). The naphthalimide ring system is

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Table II

compound	fluorescence microscopy[a] microscope filter set	fluorescence intensity hypoxic	fluorescence intensity oxic	t max, h	flow cytometry fluorescence intensity hypoxic	flow cytometry fluorescence intensity oxic	uptake,[b] fmol/cell
5	c	++	+	4	335	17	98
6	abc	++	++	6	235	42	93
7	abc	++	++	6	37	12	0.04
9	abc	-	-	4	24	13	<0.005
10					263	78	105
12					85	39	
16	a	++	-	14	412	24	
21					203	85	
22					1149	238	
23	a	++	-	3	361	24	
24					1461	64	
25	abc	-	-	6			
26	abc	-	-	6	77	19	

[a] All drug concentrations were 10^{-4} M. Band-pass excitation filters were (a) 340–380 nm, (b) 355–425 nm, (c) 450–490 nm, matched with long-pass emission filters (a) >430 nm, (b) >460 nm, (c) >515 nm, respectively. Where more than one filter set was used, each pair of excitation and emission filters was used in turn. Time of incubation of cells with drug at which maximum fluorescence was observed or longest time used if fluorescence had not peaked. Fluorescence was excited at 366 nm in the flow cytometer and all wavelengths >390 nm collected, except for compounds **5–7**, **9**, and **10**, which were excited at 458 nm and fluorescence collected in the green channel. Mean fluorescence intensities (in arbitrary units) of cells after 6-h incubation with drugs, measured by flow cytometry, have been normalized for constant PMT gain and laser-excitation power. [b] Cellular content after 4-h incubation with drugs under aerobic conditions at 37 °C.

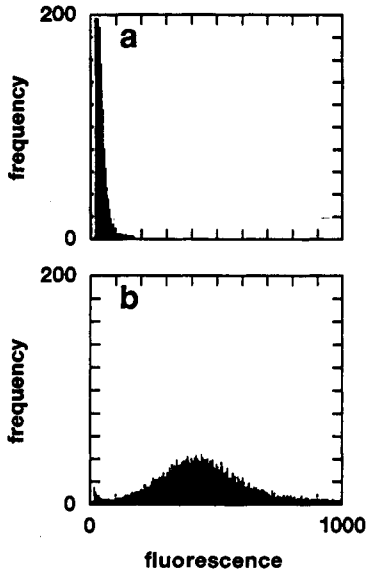

Figure 2. Flow cytometric analysis of V79 Chinese hamster cells incubated at 37 °C with **16** under (a) oxic and (b) hypoxic conditions. Data was gated on forward scatter and fluorescence to omit debris. The gated data was offset from channel 1 for presentation.

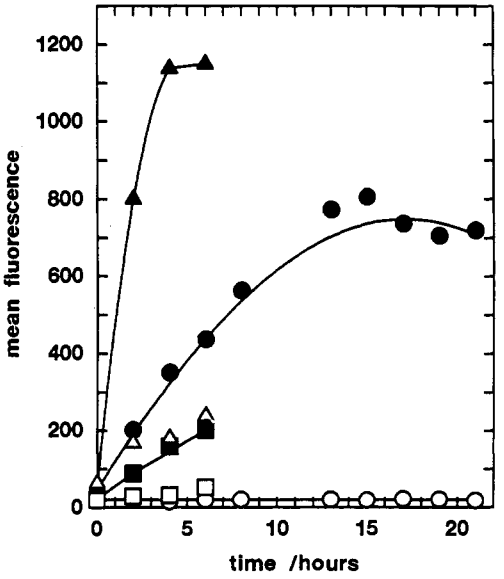

Figure 3. Typical time courses of accumulation of fluorescent metabolites in V79 Chinese hamster cells incubated with 10^{-4} M of some of the compounds from Scheme I. Mean fluorescence from hypoxic cells incubated at 37 °C with drugs: **16** (●), **21** (■), **22** (▲). Mean fluorescence from oxic cells: **16** (○), **21** (□), **22** (△).

known to intercalate into DNA,[29] leading to inhibition of DNA and RNA synthesis.[30,31] We have found previously that planar fluorescent ring systems, which can intercalate with DNA, become concentrated into cells leading to a potential microscopic bioavailability problem in vivo[19] and even higher intracellular:extracellular ratios were found with **5**, **6** and **10** compared with those reported previously for other naphthalimides. The high cellular uptakes could lead to depletion of the compound, as it diffuses through tissues in vivo, before it reaches the target hypoxic cells where it could be metabolized. By comparison, misonidazole equilibrates with cells so that an extracellular concentration of 0.1 mmol dm^{-3} leads to an average intracellular concentration of 0.07 mmol dm^{-3}.[32] The method of measuring cell uptake used for **5**, **6**, and **10** was unsuitable for other compounds which were not retained in washed cells; **9** may fall into this latter class of compound as no significant accumulation of parent compound or reduced metabolites could be detected in washed hypoxic cells; because some toxicity is seen with hypoxic cells (data not shown), it is likely that **9** can cross the cell membrane. It is possible that the long side chain on the 4-position of the naphthalimide ring interferes with this compound intercalating with DNA, thus removing a mechanism by which fluorescent reduced products can accumulate in cells.

Flow cytometric analysis of cells incubated under oxic or hypoxic conditions with compounds from Schemes I and

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II is shown in Table II and Figures 2 and 3. Most of the compounds tested exhibited some fluorescence; with several compounds (especially **5**, **6**, **16**, and **22–24**) there was a significant oxic–hypoxic differential. However, in other cases (e.g. **12** and **21**) there was little differential between the fluorescence from cells incubated with drugs under oxic and hypoxic conditions. In a few cases, the cellular oxic and hypoxic fluorescence was similar to the background fluorescence from untreated cells. The results from flow cytometry studies (Table II) showed that increasing the chain length from three carbon atoms in **5** to nine carbon atoms in **10** did not show the increase in differential fluorescence between hypoxic and oxic cells that we had thought might occur. Indeed, chain-length increases through the limited series **5**, **6**, and **10** produced a progressive decrease in the fluorescence differential while cell uptake changed little. The negligible amount of overlap between the fluorescence of hypoxic and well-oxygenated cells incubated with **16** (Figure 2) would be a desirable property of a compound used to measure a low proportion of hypoxic cells in a mainly well-oxygenated tumor in vivo.

The time course of development of fluorescent products in cells incubated with **16** is shown in Figure 3. A large differential can be seen between the fluorescence of cells incubated under oxic and hypoxic conditions. Failure of drugs to bind firmly to cellular macromolecules does not necessarily prevent the accumulation of sufficient fluorescent metabolite for analysis with a cytofluorimeter. For example, **16** is readily removed from cells by washing with PBS although its fluorescent metabolites remain. However, the majority of these fluorescent metabolites are not covalently bound to macromolecules because permeabilization of cells with Triton-X100 detergent or by fixing cells in 70% ethanol greatly reduces the amount of fluorescence observed (data not shown). Fortunately, it is relatively easy to obtain a single-cell suspension from both rodent and human tumors using enzymic digests of finely minced tumors (e.g. ref 33). With dye-exclusion and colony-forming assays, these cells appear to be intact and should therefore retain unbound fluorescent metabolites of compounds such as **16**. As an alternative approach, we have recently used immunofluorescent detection of bound metabolites of a related compound to visualize hypoxic cells in tumors exposed to the compound in vivo.[33]

Conclusions

All the target compounds tested contain an identical nitroimidazole, an electron-affinic structure, which is presumably responsible for much of the large oxic–hypoxic differential in metabolic drug binding. However, the nitroimidazole part of these drugs cannot be the only factor contributing to the observed differential in fluorescence. Surprisingly small differences between the structures of the fluorescent rings can lead to large differences in overall cellular fluorescence, e.g. the coumarins **21** vs **22**. The hypoxic–oxic differential in fluorescence also seems to be sensitive to the structure of the fluorescent ring, and the length of the carbon chain linking naphthalimides to the nitroimidazole ring has a large influence on the cellular fluorescence of bioreductively derived metabolites. Some of these differences may reflect cellular metabolism of the fluorescent parts of the molecules in addition to the bioreductive metabolism of the nitroimidazole. For example, several hypoxia-specific fluorescent metabolites have been observed after cellular metabolism of 3-nitronaphthalimides.[15,17]

The group of compounds included in this study have shown the potential of attaching fluorescent labels to 2-nitroimidazoles in the development of fluorescent markers for hypoxic cells. Some of these compounds (e.g. **5**, **6**, **16**, and **22–24**) show a 5-fold or higher hypoxic–oxic differential in the in vitro test system and may be suitable for evaluation in vivo. Of these **5** and **6** may be too easily depleted by high cellular uptake as they diffuse through packed cells in tumors and tissues to be of practical use in vivo. Thus, **16** and **22–24** may be the most promising candidates for detecting hypoxic cells in tumors in vivo.

Experimental Section

Chemistry. Melting points were recorded on a Mettler FP61 instrument and are uncorrected. Elemental analyses were performed by Medac Ltd., Brunel University, on a CEC Model 240XA analyzer. Infrared spectra were recorded for films or potassium bromide disks on a Pye-Unicam SP3-100 spectrometer or a Perkin-Elmer 1420 instrument. Proton magnetic resonance spectra were recorded on a Varian T-60 or CFT-20 spectrometer or a JEOL FX-200 instrument for solutions in DMSO-d_6, unless otherwise specified, containing tetramethylsilane as internal standard. Electron impact mass spectra were obtained on an EMI MS902 double-focusing spectrometer.

Thin-layer chromatography was carried out on silica gel of 0.25-mm layer thickness. Column chromatography was performed on Kieselgel 60 of 70–230 or 230–240 mesh ASTM. Preparative high-performance chromatography was achieved with a Gilson modular autoprep instrument with silica gel (5-μm particle diameter) packing in the column (30 cm × 21.4 mm i.d.). Solvents were distilled before use. Light petroleum refers to the fraction boiling at 40–60 °C, and ether refers to diethyl ether.

Ultraviolet absorption and visible fluorescence spectra were obtained at 25 °C with a Pye-Unicam SP8-200 and Perkin-Elmer LS-5 spectrometer, respectively, the latter fitted with a red-sensitive R928 photomultiplier. Fluorescence emission spectra were recorded with 10-nm excitation and emission bandwidth for methanolic solution with optical densities in the range 0.05–0.1 at the excitation wavelength in silica cells (10-mm pathlength). Emission correction factors were determined at 10-nm intervals from 250 to 750 nm by reference to an excitation beam diffuser plane (Perkin-Elmer 5212-4054) and quinine sulfate dihydrate in 0.1 M perchloric acid. A computer program which incorporated the correction factors reported by Lippert et al.[34] was used to obtain corrected emission spectra and to calculate the quantum yield with quinine sulfate (0.4 μM) in perchloric acid (0.1 M) as the standard.[35]

4-Amino-*N*-(3-bromopropyl)naphthalimide (**1**), 4-acetamido-*N*-(3-bromopropyl)naphthalimide (**2**),[21] 1-(2,3-epoxypropyl)-2-nitroimidazole (**13**),[25] and ethyl 7-methoxycoumarin-3-carboxylate (**14**)[26] were prepared by literature methods. 4-Aminonaphthalimide, 4-(bromomethyl)-7-methoxycoumarin, 7-methoxy-3-methylcoumarin and 6,7-dimethoxy-3-methylcoumarin were obtained from the Aldrich Chemical Co. Azomycin was a gift from Roche Products Ltd., U.K., or was purchased from Fluka Chemicals, U.K.

4-Amino-*N*-(ω-bromoalkyl)-1,8-naphthalimides (3 and 4). General Method. Sodium methoxide solution in methanol [2.76 mL of a solution of sodium (2 g) in methanol (10 mL), 24 mmol] was added dropwise to a stirred solution of 4-amino-1,8-naphthalimide (5 g, 23.6 mmol) in DMF (150 mL) at room temperature. The red solution was stirred for a further 15 min and then the 1,ω-dibromoalkane (95 mmol) was added in one portion. The dark solution was allowed to stand for 1 h and then poured into ice–water (ca. 1500 mL) to give a yellow solid which was filtered off and dried.

4-Amino-*N*-(5-bromopentyl)-1,8-naphthalimide (3) was obtained (89%) by crystallization from HOAc: mp 157–159 °C dec; NMR δ 1.42–1.94 (m, 6 H, 3 × CH_2), 3.53 (t, 2 H, CH_2Br),

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4.00 (t, 2 H, CH_2N), 6.82 (d, 1 H, 3-H), 7.34 (s, 2 H, NH_2), 7.61 (t, 1 H, 6-H), and 8.11–8.63 (m, 3 H, 2-, 5-, and 7-H). Anal. ($C_{17}H_{17}BrN_2O_2$) C, H, N.

4-Amino-*N*-(9-bromononyl)-1,8-naphthalimide (4) was isolated (68%) by crystallization from HOAc. An analytical sample was obtained by preparative HPLC using EtAc as the eluant: mp 124–125 °C; NMR δ ($CDCl_3$) 1.32–1.91 (m, 14 H, 7 × CH_2), 3.36 (t, 2 H, CH_2Br), 4.13 (t, 2 H, CH_2N), 4.88 (br s, 2 H, NH_2), 6.84 (d, 1 H, 3-H), 7.60 (m, 1 H, 6-H), 8.05 (dd, 1 H, 5-H), 8.37 (d, 1 H, 2-H), and 8.55 (dd, 1 H, 7-H); MS *m/z* 418 (11, M^+), 416 (11, M^+), and 212 (100). Anal. ($C_{21}H_{25}BrN_2O_2$) C, H, N.

Typical Procedure for the N-Alkylation of Azomycin with *N*-(Bromoalkyl)naphthalimides. Sodium methoxide (1.45 g, 27 mmol) in methanol (12 mL) was added dropwise to a slurry of 2-nitroimidazole (3 g, 26 mmol) in DMF (60 mL). The resulting solution was heated at 150 °C to boil off the methanol and then the appropriate 4-substituted-*N*-(ω-bromoalkyl)-1,8-naphthalimide (26 mmol) in DMF (60 mL) was added at 140 °C and the mixture maintained at that temperature for 15 min. The cooled reaction mixture was poured onto crushed ice, the solid was filtered off and washed with water. This solid was dissolved in the minimum of hot DMF-EtOH mixture (1:1, v/v) and the warm solution filtered. Water was added to the filtrate and the crude product filtered off at the pump.

4-Amino-*N*-[3-(2-nitroimidazol-1-yl)propyl]-1,8-naphthalimide (5). The crude product was crystallized from aqueous DMF (charcoal) to provide the naphthalimide derivative (4.37 g, 46%): mp 261–263 °C; NMR δ 2.16 (q, 2 H, *J* = 7 Hz, $CH_2CH_2CH_2$), 4.07 (t, 2 H, *J* = 7 Hz, naphth-CH_2), 4.46 (t, 2 H, *J* = 7 Hz, imidazole-CH_2), 6.83 (d, 1 H, *J* = 9 Hz, 3-H), 7.14 (d, 1 H, *J* = 2 Hz, 4′-H), 7.62 (m, 1 H, 6-H), 8.11 (d, 1 H, *J* = 2 Hz, 5′-H), 8.29 (d, 1 H, *J* = 9 Hz, 2-H), 8.40 (dd, 1 H, *J* = 2 and 9 Hz, 5-H), and 8.59 (dd, 1 H, *J* = 2 and 9 Hz, 7-H); MS *m/z* 335 (2, M^+), 319 (47, $M^+ - NO_2$), and 212 (100). Anal. ($C_{18}H_{15}N_5O_4$) C, H, N.

4-Acetamido-*N*-[3-(2-nitroimidazol-1-yl)propyl]-1,8-naphthalimide (7). Crystallization of the crude product from aqueous DMF afforded to product (78%): mp 222–223 °C. Anal. ($C_{20}H_{17}N_5O_5$) C, H, N. The same compound was obtained by acetylation of **5** in a mixture of HOAc and Ac_2O.

4-Amino-*N*-[3-(2′-nitroimidazol-1′-yl)pentyl]-1,8-naphthalimide (6) was crystallized from aqueous HOAc (70%) to give a 67% yield: mp 202–203 °C; IR ν (KBr) 3420 and 3350 (NH_2), 3250 (CH_2), 1675 and 1650 (CO), and 1560 and 1350 cm^{-1} (NO_2); NMR δ 1.47–1.93 (m, 6 H 3 × CH_2), 4.00 (t, 2 H, CH_2N), 4.37 (t, 2 H, CH_2Br), 6.82 (d, 1 H, 3-H), 7.11 (s, 1 H, 5′-H), 7.37 (br s, 2 H, NH_2), and 7.51–8.62 (m, 5-H, 2-, 5-, 6-, 7-, and 4′-H). Anal. ($C_{20}H_{19}N_5O_4$) C, H, N.

4-Amino-*N*-[9-(2-nitroimidazol-1-yl)nonyl]-1,8-naphthalimides (10). Preparative HPLC with EtOAc as eluent gave **8** (68%): mp 143–143 °C. Anal. ($C_{24}H_{27}N_5O_4$) C, H, N.

4-(Chloroacetamido)-*N*-[3-(2-nitroimidazol-1-yl)-propyl]-1,8-naphthalimide (8). A mixture of **5** (2 g), chloroacetic anhydride (4 g), and chloroacetic acid (20 g) was heated at 70 °C for 1 h. The hot reaction mixture was poured onto ice and the resultant light yellow solid was filtered off and washed with water to afford **8** (2.27 g, 96%): mp > 300 °C; IR ν (KBr) 1695 (chloroacetamide CO) and 1650 cm^{-1} (imide CO); NMR δ 2.19 (q, 2 H, $CH_2CH_2CH_2$), 4.09 (t, 2 H, napth-CH_2), 4.38–4.55 (m, 4 H, imidazole-CH_2 and $COCH_2Cl$), 7.14 (d, 1 H, 4′-H), 7.72 (d, 1 H, 5′-H), 7.91 (d, 1 H, 3-H), 8.13–8.66 (m, 4 H, 2-, 5-, 6-, and 7-H), and 10.63 (br s, 1 H, NH). Anal. ($C_{20}H_{16}ClN_5O_5$) C, H, N.

***N*-[3-(2-Nitroimidazol-1-yl)propyl]-4-[[[3-[bis-(2-hydroxyethyl)amino]propyl]amino]acetamido]-1,8-naphthalimide Trihydrochloride (9).** *N,N*-Bis(2-hydroxyethyl)propane-1,3-diamine (200 mg, 1.23 mmol), **8** (200 mg, 0.45 mmol), and DMF (2 mL) were stirred together for 3 h. The mixture was then poured into ice–water (ca. 25 mL) and the resultant gelatinous solid was filtered off, dried, and dissolved in ethanol (25 mL). Dry HCl was passed into the solution and the precipitated **9** collected (170 mg, 61%): mp 130 °C dec; NMR δ 1.14 (q, 4 H, *J* = 7 Hz, 2 × $CH_2CH_2CH_2$), 3.02–4.56 (m, 20 H, which changed to 18 H on D_2O exchange, 2 × CH_2CH_2OH, $COCH_2NHCH_2CHCH_2$, naphth-CH_2, and imidazole-CH_2), 7.14 (s, 1 H, 4′-H), 7.73 (s, 1 H, 5′-H), 7.87 (t, 1 H, 6-H), 8.21 (d, 1 H, 3-H), 8.50 (d, 2 H, 2- and 5-H), 8.83 (d, 1 H, 7-H), 9.48 (br s, 3 H, $COCH_2NH_2^+$ and NH^+), 9.96 (br s, 1 H, $NH(CH_2CH_2OH)_2$) and 11.23 (br s, 1 H, CONH). Anal. ($C_{27}H_{36}Cl_3N_7O_7$) C, H, N.

1-(6,7-Dimethoxy-4-aminoquinazol-2-yl)-4-[2-hydroxy-3-(2-nitroimidazol-1-yl)propyl]piperazine (12). A mixture of **13** (444 mg, 1.54 mmol), **11** (200 mg, 1.18 mmol), and MeOH (3 mL) was heated at reflux for 18 h. Evaporation of the solvent under reduced pressure and crystallization of the residue from EtOH afforded **12** (248 mg, 46%): mp 196–198 °C; NMR δ 3.25 (s, 8 H, 2 × CH_2CH_2), 3.76 (s, 3 H, OCH_3), 3.80 (s, 3 H, OCH_3), 3.87–4.13 (m, 1 H, CHOH), 4.35 (d, 2 H, NCH_2), 4.66 (d, 2 H, NCH_2), 5.07 (br s, 1 H, OH), 6.68 (s, 1 H, 5-H), 7.00 (br s, 2 H, NH_2), 7.11 (d, 1 H, 4′-H), 7.36 (s, 1 H, 8-H), and 7.54 (d, 1 H, 5′-H). Anal. ($C_{20}H_{26}N_8O_5$) C, H, N.

***N*-[2-[(2-Hydroxyethyl)amino]ethyl]-7-methoxycoumarin-3-carboxamide (15).** A mixture of ethyl 7-methoxycoumarin-3-carboxylate (10 g, 40 mmol), 2-(2-aminoethylamino)ethanol (10 g, 96 mmol), and EtOH (80 mL) was stirred at room temperature for 18 h and then cooled to -20 °C for 2 h. The resultant pale yellow solid was filtered off and crystallized from EtOH to afford amide **15** (2.9 g, 24%): mp 144–145 °C; IR ν (KBr) 3600–2400 (OH), 3340 and 3300 (amide and amine NH) and 1710 cm^{-1} (amide and coumarin CO): NMR δ 2.50 (m, 4 H, 2 × CH_2), 2.71 (m, 4 H, 2 × CH_2), 3.91 (s, 3 H, CH_3), 7.00 (s and dd, 6- and 8-H), 7.91 (d, 1 H, 5-H), and 8.84 (s and br s, 2 H changed to 1 H on addition of D_2O, 4 H and NH or OH). Anal. ($C_{15}H_{18}N_2O_5$) C, H, N.

***N*-[2-[*N*′-(2-Hydroxyethyl)-*N*′-[3-(2-nitroimidazol-1-yl)-2-hydroxypropyl]amino]ethyl]-7-methoxycoumarin-3-carboxamide (16).** A mixture of the aforementioned amide **15** (3.5 g, 11.4 mmol), 1-(2,3-epoxypropyl)-2-nitroimidazole (1.41 g, 8.4 mmol), and MeOH (11 mL) was heated at reflux for 14 h. The mixture was cooled to −20 °C; the solid was removed and recrystallized from MeOH (with charcoal) to give product **16** (1.72 g, 43%): mp 140–141 °C; IR ν (KBr) 1350 cm^{-1} (NO_2); NMR δ 2.62 (t, 6 H, $NCH_2CH_2CH_2$), 3.31–3.51 (m, 6 H, $CONHCH_2$, CH_2OH, and imidazole-CH_2), 3.88 (s, 3 H, OCH_3), 4.02–4.99 (m, 3 H which changes to 1 H on addition of D_2O, CHOH and 2 × OH), 6.92–7.04 (m, 2 H, 6- and 8-H), 7.10 (s, 1 H, 4-H of imidazole), 7.52 (s, 1 H, 5-H of imidazole), 7.84 (d, 1 H, 5-H), and 8.76 (br s, 2 H which changes to 1 H on addition of D_2O, CONH, and 4-H). Anal. ($C_{21}H_{25}N_5O_8$) C, H, N.

7-Methoxy-3-methylcoumarin (17). 3-Hydroxyanisole (1.245 g, 10 mmol), ethyl 3,3-diethoxy-2-methylpropionate (1.48 g, 7.25 mmol), and phosphoric acid (10 mL, 85%) were heated together at 100 °C for 1 h. The reaction mixture was poured onto crushed ice (ca. 500 mL) and the crude solid filtered off. Column chromatography of the crude product using light petroleum ethyl acetate (3:1) as eluent afforded the coumarin (430 mg, 30%): mp 136–137 °C; NMR δ ($CDCl_3$) 2.16 (d, *J* = 2 Hz, 3 H, CH_3), 3.83 (s, 3 H, OCH_3), 6.69–6.86 (m, 2 H, 6- and 8-H), 7.21 (d, *J* = 2 Hz, 1 H, 4-H), and 7.35 (dd, 1 H, 5-H); MS *m/z* 190 (M^+, 100%), 162 (33), and 147 (74). Anal. ($C_{11}H_{10}O_3$) C, H.

6,7-Dimethoxy-3-methylcoumarin (18). In a similar way to that used for the preparation of **17**, 3,4-dimethoxyphenol yielded a crude solid which was crystallized from EtOH (with charcoal) to give title compound **18** (45%): mp 127–128 °C; NMR δ ($CDCl_3$) 2.17 (d, *J* = 2 Hz, 3 H, CH_3), 3.88 (s, 3 H, OCH_3), 3.90 (s, 3 H, OCH_3), 6.76 (s, 1 H, 8-H), 6.78 (s, 1 H, 5-H), and 7.38 (d, 1 H, *J* = 2 Hz, 4-H); MS *m/z* 220 (M^+, 100), 205 (34), and 177 (24). Anal. ($C_{12}H_{12}O_4$) C, H.

3-(Bromomethyl)-7-methoxycoumarin (19). 7-Methoxy-3-methylcoumarin (2.0 g, 10.5 mmol), *N*-bromosuccinimide (1.87 g, 10.5 mmol), dibenzoyl peroxide (20 mg), and carbon tetrachloride (150 mL) were heated together at reflux under nitrogen for 2.5 h while being irradiated with a tungsten-filament lamp. Succinimide was filtered off from the cold reaction mixture and the filtrate evaporated to dryness. Column chromatography of the residue using light petroleum–ethyl acetate as eluent gave a solid which was crystallized from ethanol to give product **19** (210 mg, 8%): mp 131–132 °C; NMR δ ($CDCl_3$) 3.85 (s, 3 H, OCH_3), 4.39 (s, 2 H, CH_2Br), 6.76–6.89 (m, 2 H, 6- and 8-H), 7.35 (d, 1 H, 5-H), and 7.73 (s, 1 H, 4-H); MS *m/z* 270 (M^+, 4), 268 (M^+, 4), and 189 (100). Anal. ($C_{11}H_9BrO_3$) C, H.

3-(Bromomethyl)-6,7-dimethoxycoumarin (20). In a similar way to the preparation of **19**, 6,7-dimethoxy-3-methylcoumarin

yielded a crude product which was purified by preparative HPLC using hexane–propan-2-ol mixture as eluent to give **20** (530 mg, 39%): mp 182–184 °C; NMR δ ($CDCl_3$) 3.89 (s, 3 H, OCH_3), 3.92 (s, 3 H, OCH_3), 4.41 (d, J = 2 Hz, 2 H, CH_2Br), 6.81 (s, 1 H, 8-H), 6.82 (s, 1 H, 5-H), and 7.73 (d, J = 2 Hz, 1 H, 4-H). Anal. ($C_{12}H_{11}BrO_4$) C, H.

N-Alkylation of 2-Nitroimidazole by (Bromomethyl)coumarins. General Method. Sodium methoxide in methanol was added to a solution of 2-nitroimidazole (15 mmol) in DMF (30 mL) until an orange end point was observed. The mixture was heated to 140 °C and a solution of (bromomethyl)coumarin (15 mmol) in DMF (50 mL) was added and the mixture heated at 150 °C for a further 1 h for the 4-(bromomethyl)coumarins or 5 h for the 3-(bromomethyl)coumarins. The reaction mixture was then poured into crushed ice (ca. 1 L) and the crude product either collected by filtration (for the 4-substituted coumarins) or extracted into ethyl acetate (for the 3-substituted coumarins) and the solution evaporated.

7-Methoxy-3-(2-nitroimidazol-1-yl)methylcoumarin (21). The crude solid was crystallized from dichloromethane to give yellow product **21** (54%): mp 172–174 °C; NMR δ ($CDCl_3$) 3.85 (s, 3 H, OCH_3), 5.45 (d, 2 H, NCH_2), 6.77–6.90 (m, 2 H, 6- and 8-H), 7.12 (d, 1 H, 4-H of imidazole), 7.38 (dd, 1 H, 5-H), 7.48 (d, 1 H, 5-H of imidazole), and 7.78 (d, 1 H, 4-H); MS m/z 301.07016, $C_{14}H_{11}N_3O_5$ requires M^+ 301.06986.

6,7-Dimethoxy-3-(2-nitroimidazol-1-yl)methylcoumarin (22). Crystallization of the crude solid from dichloromethane gave yellow **22** (46%): mp 174–176 °C; NMR δ ($CDCl_3$) 3.89 (s, 3 H, OCH_3), 3.92 (s, 3 H, OCH_3), 5.46 (d, 2 H, NCH_2), 6.79 (s, 1 H, 8-H), 6.84 (s, 1 H, 5-H), 7.13 (d, 1 H, 4-H of imidazole), 5.70 (d, 1 H, 5-H of imidazole), and 7.78 (d, 1 H, 4-H); MS m/z 331.080765, $C_{15}H_{13}N_3O_6$ requires M^+ 331.08043.

7-Methoxy-4-(2-nitroimidazol-1-yl)methylcoumarin (23). The crude solid was crystallized from EtOH-HOAc (4:1) to give yellow **23** (75%): mp 222–245 °C; NMR δ 3.88 (s, 3 H, OCH_3), 5.35 (d, 1 H, 4-H), 5.90 (d, 2 H, NCH_2), 6.95–7.07 (m, 2 H, 6- and 8-H), 7.31 (d, 1 H, 4-H of imidazole), 7.73 (d, 1 H, 5-H of imidazole), and 7.75 (d, 1 H, 5-H). Anal. ($C_{14}H_{11}N_3O_5$) C, H, N.

6,7-Dimethoxy-4-(2-nitroimidazol-1-yl)methylcoumarin (24). Preparative TLC on silica gel using ethyl acetate as eluent and crystallization from EtOH gave coumarin **24** (19%): mp 214 °C dec; NMR δ 3.87 (s, 3 H, OCH_3), 3.90 (s, 3 H, OCH_3), 5.32 (d, 1 H, 3 H), 5.95 (d, 2 H, NCH_2), 7.14 (s, 1 H, 8-H), 7.23 (s, 1 H, 5-H), 7.34 (d, 1 H, 4-H of imidazole), and 7.75 (d, 1 H, 5-H of imidazole). Anal. ($C_{15}H_{13}N_3O_6$) C, H, N.

7-Methoxy-4-[[(4-nitrobenzoyl)oxy]methyl]coumarin (25). 4-(Bromomethyl)-7-methoxycoumarin (500 mg, 1.77 mmol), 4-nitrobenzoic acid (300 mg, 1.80 mmol), anhydrous potassium carbonate (5.0 g), 18-crown-6 (250 mg), and acetone (500 mL) were heated together at reflux under a calcium chloride guard tube in the dark for 1.5 h. The hot reaction mixture was filtered and the acetone evaporated to leave the crude product which was crystallized from a EtOH–DMF mixture to give ester **25** (360 mg, 57%): mp 226–228 °C; IR ν (KBr) 1740 (ester CO) and 1715 cm^{-1} (lactone CO); NMR δ 3.87 (s, 3 H, OCH_3), 5.52 (d, 2 H, CH_2O), 6.37 (d, 1 H, 3-H), 6.81–6.93 (m, 2 H, 6- and 8-H), 7.45 (d, 1 H, 5-H), and 8.25 (s, 4 H, $C_6H_4NO_2$); MS m/z 355 (M^+, 26), 151 (18), 150 (100), and 120 (50). Anal. ($C_{18}H_{13}NO_7$) C, H, N.

4-[[(2-Aminoethyl)thio]methyl]-7-methoxycoumarin Hydrobromide (26). A mixture of 4-(bromomethyl)-7-methoxycoumarin (0.81 g, 3 mmol), 2-aminoethanethiol (0.24 g, 3 mmol), and dry acetonitrile (70 mL) was heated under reflux for 0.5 h. A solid was deposited and this was filtered off from the warm reaction mixture and recrystallized from EtOH as amine **26** (0.55 g, 69%): mp 220–221 °C; IR ν (KBr) 3450 and 3405 (NH_2) and 1700 cm^{-1} (CO); NMR δ 2.65–3.01 (m, 4 H, 2 × CH_2), 3.84 (s, 3 H, OCH_3), 3.98 (s, 2 H, CH_2S), 6.36 (s, 1 H, 3-H), 6.92 (dd, 1 H, 6-H), 6.98 (d, 1 H, 8-H), 7.67 (br s, 2 H, NH_2), and 7.76 (d, 1 H, 5-H). Anal. ($C_{13}H_{16}BrNO_3S$) C, H, N.

7-Hydroxy-1-(7-methoxycoumarin-4-yl)-8-(2-nitroimidazol-1-yl)-5-aza-2-thiaoctane (27). Amine hydrobromide **26** (159 mg, 0.46 mmol), azomycin epoxide, **13** (118 mg, 0.7 mmol), and MeOH (30 mL) were refluxed together for 16 h in subdued light. The solvent was evaporated and the residue separated by column chromatography using ethyl acetate as eluent to give **27** (64 mg, 32%) which was crystallized from MeOH: mp 159–160 °C dec; IR ν (KBr) 3450 (OH), 3305 (NH), 1730 and 1720 (CO), and 1522 and 1350 cm^{-1} (NO_2); MS m/z (FAB) 435 (M^+ + 1) and 405 (M^+ + 1 − NO). Anal. ($C_{19}H_{22}N_4O_6S$) C, H, N.

Biology. Because of the poor water solubility of some of the compounds used in this work, drugs were initially dissolved at 10–20 mmol dm^{-3} in dimethyl sulfoxide (DMSO) and small volumes added to cell suspensions to give the appropriate drug concentration. The final concentration of DMSO was 1% or less. DMSO and other reagents were BDH AnalaR grade. Special gases (air + 5% CO_2, nitrogen + 5% CO_2) were obtained from British Oxygen Co.

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% fetal calf serum. The method of incubating cells with drugs for measuring the production of fluorescent products has been described.[17,19] Fluorescence microscopy was used to evaluate samples of cells incubated with some of the compounds under aerobic or hypoxic conditions; these observations were used as a guide for further evaluation by flow cytometry.

Flow cytometric analysis of cells for fluorescent products was carried out in an Ortho Systems 50 cytofluorograph using a Coherent 5W laser. For compounds other than those with a naphthalimide fluorescent label, excitation was at 365 nm with an output of 100–200 mW. 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 cutoff filter that passed all wavelengths above 390 or 410 nm (Table II). For compounds with naphthalimide fluorophors (**5**, **6**, **7**, **9**, and **10**), excitation was at 458 nm and green fluorescent emission was collected between 510 and 560 nm. Forward-scattered light at the excitation wavelength was also collected and used to discriminate noncellular debris (low scatter).

The method of preparing cell samples for drug-uptake studies has been described.[19] The HPLC equipment and eluents were similar to those described by Hodgkiss et. al.[19], except that a Waters 481 variable-wavelength detector was used. For **6**, **5**, and **10** a linear gradient from 50 to 75% acetonitrile in 10 min was used, with detection at 390 nm. For **7** and **9**, the gradient was from 30 to 50% in 10 min, with detection at 340 nm.

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Registry No. **3**, 133932-09-9; **4**, 133932-10-2; **5**, 133932-11-3; **6**, 133932-12-4; **7**, 133932-13-5; **8**, 133932-14-6; **9**·3HCl, 133932-15-7; **10**, 133932-16-8; **11**, 60547-97-9; **12**, 133932-17-9; **13**, 13551-90-1; **14**, 6093-72-7; **15**, 133932-18-0; **16**, 133932-19-1; **17**, 72136-39-1; **18**, 86818-97-5; **19**, 133932-20-4; **20**, 133932-21-5; **21**, 133932-22-6; **22**, 133932-23-7; **23**, 133932-24-8; **24**, 133932-25-9; **25**, 133932-26-0; **26**·HBr, 133932-27-1; **27**, 133932-28-2; OH-*m*-C_6H_4-OMe, 150-19-6; $(EtO)_2CHCH(CH_3)CO_2Et$, 36056-90-3; NO_2-*p*-C_6H_4-CO_2H, 62-23-7; 4-amino-1,8-naphthalimide, 1742-95-6; 2-nitroimidazole, 527-73-1; 3,4-dimethoxyphenol, 2033-89-8.