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ORIGINAL PAPER

Versatile Nitro-Fluorophore as Highly Effective Sensor for Hypoxic Tumor Cells: Design, Imaging and Evaluation

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Abstract Based on the characteristics of a nitro-group, which can be partially or totally reduced to an amino-group in hypoxic cells, two series of hypoxic sensors containing 7H-Benzimidazole [2,1-a]benz[de]isoquinolin-7-one cores with nitro groups in different positions were designed, synthesized and evaluated. The target compounds exhibited significantly different fluorescence characteristics (fluorescence enhancement or quenching). The strong fluorescences of some partially reduced compounds could be explained through the examination of their infrared spectra, which showed a restriction of the nitro-group vibration. These compounds exhibited significant hypoxic–oxic fluorescence differences not only in numerical values but also in their fluorescent imaging properties as reported for the first time.

Keywords Nitro-fluorophore · Sensor · Hypoxic · Tumor cell

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Introduction

Tumor hypoxia has been linked to the unsuccessful outcome of therapeutic treatments, especially radiotherapy [1, 2]. Chemotherapy is also negatively affected in hypoxic cells due to the requirement for molecular oxygen in the toxicity of many anticancer agents [3]. Subsequently, there is increasing interest in the detection of hypoxic cell fractions in tumors so that optimal treatment schedules could be devised for individual patients. Of the various methods, one approach to the identification of hypoxic cells has been taking advantage of the reductive metabolism of fluorescent nitroaromatic compounds in hypoxic cells [4, 5]. Numerous nitroaromatic structures have been evaluated in model systems in vitro in numerical fluorescence values. The use of these agents as fluorescent markers was based on the following principle: the nitro-group usually quenches the fluorescence of the aromatic ring system but strong fluorescence enhancement could be noted after bioreduction in hypoxic cells. However, it should be stressed that the design and synthesis of most of the known sensors relies on the expensive 2-nitroimidazole, which is indirectly connected to the fluorophore via an alkyl bridge and is only used as fluorescent quencher[6–10]. Except that Hodgkiss's group ever reported some hypoxic markers of 3-nitronaphthalimides with alkyl chains designed to produce increased water solubility and nitro acridine derivatives showed differential effects between hypoxic and oxic cells [11, 12], almost no successful attempt to develop fluorescent molecular sensors for hypoxic cells with the nitro-group directly conjugated to fluorophore was reported. The possible reason is that the nitro group usually plays a role of fluorescence quencher (except if the absorption wavelength of the nitro-compound is above 500 nm) and nitro-

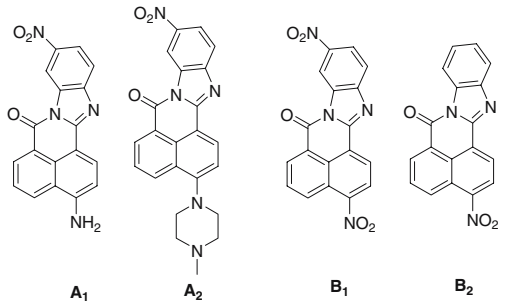

Fig. 1 The chemical structures of the target sensors

compounds as fluorophores or fluorescent molecular sensors have not often been considered.

In this paper, we will report about novel fluorescent molecular sensors for hypoxic cells containing a nitro-group directly on the fluorophore, without 2-nitroimidazole moiety. Moreover, their hypoxic–oxic fluorescence differentials are presented for the first time not only in numerical values but also in form of fluorescent images. We will also stress that the nitro-group not only could be serve as fluorescence quencher but also is helpful for fluorescence enhancement, which depends on the substitution site and vibration amplitude of nitro groups. This observation might be also useful for the design of the other fluorescent molecular sensors. Here, the active nitro-group could be easily introduced into the fluorophore through simple synthesis, and the nitro-groups act as magicians in chemical environment and cells (fluorescent quenching sensors for hypoxic cells: A series, and fluorescent enhancement sensors for hypoxic cells: B series. Fig. 1) compared to the corresponding amino derivatives.

Results and discussion

Synthesis

The chemical structures of the designed sensors were shown in Fig. 1. All the compounds were obtained by condensation of 4-substituted naphthalic anhydride and ortho-diamines as shown in Scheme 1. Their structures were confirmed by ^{1}H NMR, HRMS, and IR.

The fluorescent behaviour of nitro-fluorophores in chemical solvents

We believe that the strength of ICT effect and excited energy losing via group vibration caused by nitro moiety, which depends on the substitution position and the numbers of nitro moiety on fluorophore ring, will strongly influence the fluorescence behaviour. It was found from Table 1 that the maximal absorption wavelengths of these two series of nitro-heterocycles were lower than 500 nm. It has been reported that the nitro group is an efficient fluorescence quencher if the absorption wavelength is below 500 nm (only a few exceptions such as NBD have been reported) [13]. In our case, the compounds A_1, A_2 exhibit high fluorescent quantum yield. In order to study this surprising phenomenon, we synthesized a series of 4, 10-substituted derivatives of 7H-Benzimidazole[2,1-a]benz[de] iso-quinolin-7-ones using the same procedures as for A_1. The corresponding reduced compounds 1, 2, 3 were also synthesized by literature methods [14].

Figure 2 indicates that in case of the nitro-group connected with a benzoimidazole ring, the compounds showed strong fluorescences, (e.g. compound A_1), however,

Scheme 1 The synthesis route of the target compounds

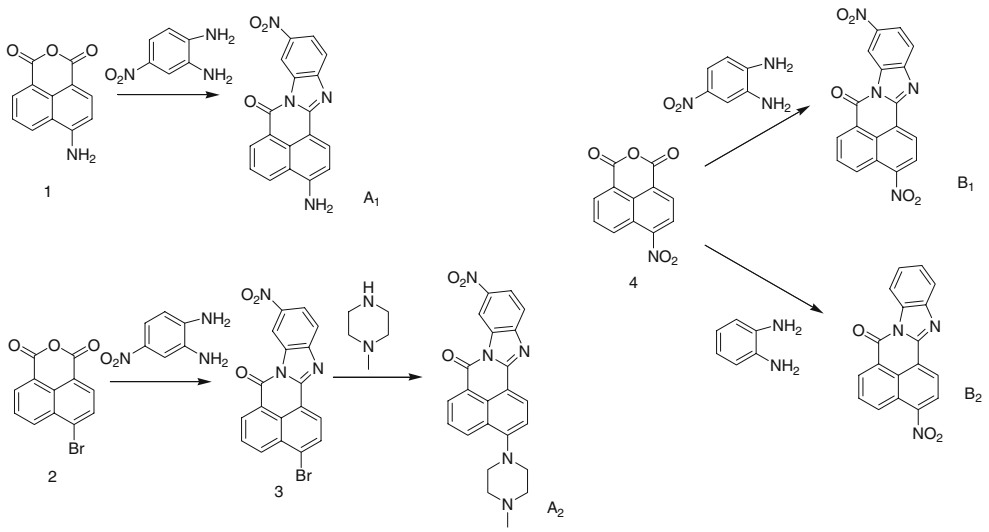

Table 1 Spectra data of compounds A series and B series[a,b]

Compound	UV, λmax (nm)	(logε)	FL, λmax (nm; Φ)
A_1	465	(4.63)	520 (0.45)
A_2	412	(4.52)	500 (0.36)
B_1	421	(4.56)	None
B_2	415	(4.51)	None
1	446	(4.21)	550 (0.36)

[a] In absolute methanol
[b] With quinine sulfate in sulfuric acid as quantum yield standard

in case of the nitro-group connected with a naphthalene ring, the compounds have no fluorescences (e.g. compound B_2). When the nitro-group was reduced to the corresponding amino-group, compound 1 (from B_2) showed strong emission while compound 3 (from B_1) showed very weak fluorescence. We speculated that, in the case of compound B_2, in which the nitro-group was connected to the naphthelene ring (the fluorophore), a very poor ICT system was formed. This might mainly act as normal fluorescent quencher due to the loss of excitation energy by efficient intersystem crossing(the quantum yield is 0.83 for 2-nitronaphthalene in benzene). But for compound A_1, the nitro-group mainly acted as an electron-withdrawing group, that is, it undergoes a strong intramolecular charge transfer (ICT) process to exhibit strong fluorescence. The maximum λ_{em} of A_1 with the very polar ICT state, shows a strong red-shift about 60 nm compared to 1. In this case the efficient intersystem crossing of the nitro-group for deactivation might be inhibited. In addition, the emission spectrum of A_1 shows fine structures on its curve, as A_1 is hydrophobic and exhibits only very poor solubility in absolute methanol. When the two nitro-groups of B_1 were reduced to the two amino-groups for formation of 3, the ICT effect of the latter was still very weak, and the fluorescence intensity was also very low. Obviously, in case of compound 2, it almost does not have strong ICT effect and the nitro-group might also consume too much excited energy to give fluorescence.

As we know, the nitro-group acts mostly as a fluorescence quencher due to its non-radiative transfer which consume excited energy by strong vibration of this group. In order to prove the above mentioned speculation, the infrared spectra of the compounds were measured. In the FT-IR spectra of A_1, B_1, B_2, 2 (Fig. 3) the stretching vibration of *C*=O were observed as sharp and strong absorption bands appearing at 1,650–1,710 cm^{-1} which could be considered as internal reference standard. The absorption bands at 1,520–1,590 cm^{-1} were assigned to the anti-stretching vibration (ν_{as}) of the nitro-group. The strong bands at 1,300–1,370 cm^{-1} were characteristics of the stretching vibration (ν_s) of the nitro-group. The peak area could reflect the energy of the vibration.

As we can see (Table 2), compared to the corresponding stretching vibration of *C*=O, the peak area (A) of the bands representing ν_{as} or ν_s of nitro-group in A_1 is less than those

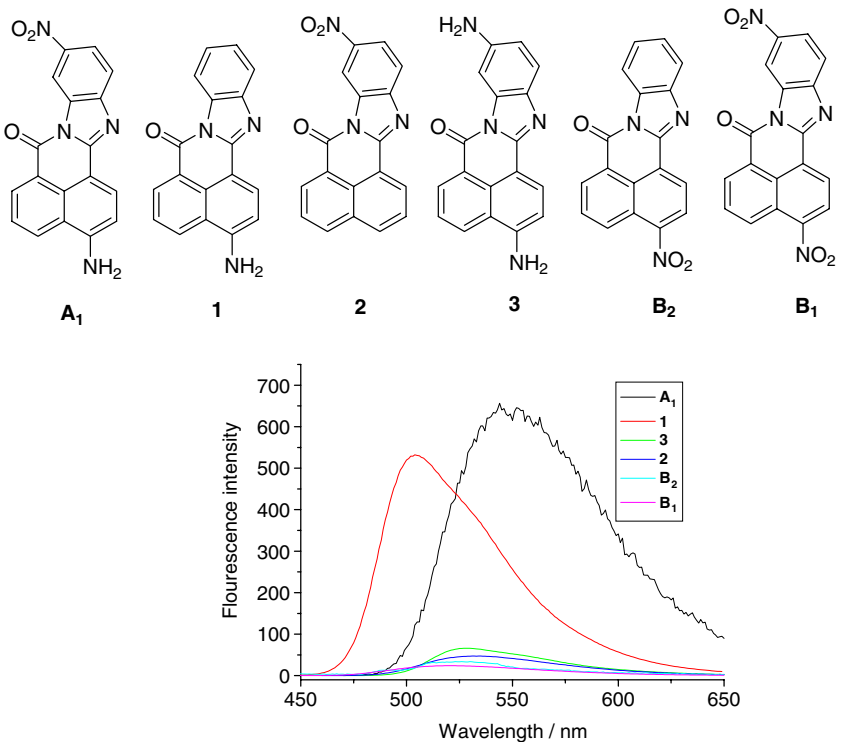

Fig. 2 Fluorescence intensities of compounds A_1, 1, 2, 3, B_2, B_1 at the concentration of 10^{-5} mol/L in absolute methanol

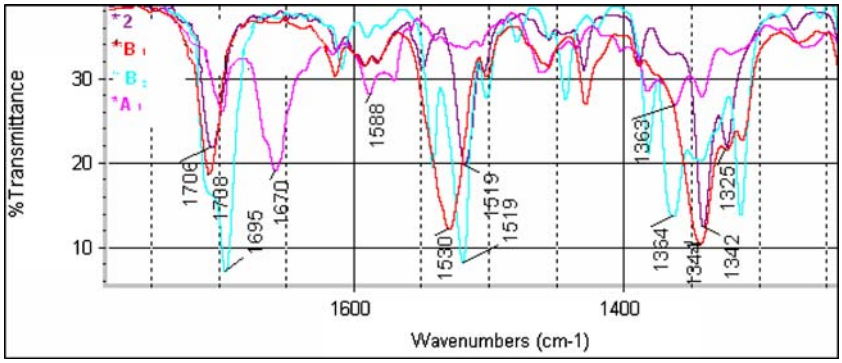

Fig. 3 FT-IR spectra of A_1, B_1, B_2, 2

of the other three compounds (especially compound 2 without electron-donating amino-group) which showed that the nitro-group in A_1 was conjugated effectively with the 7H-Benzimidazole [2,1-a]benz[de]isoquinolin-7-one moiety, it resulted in a restriction for the vibration of the nitro-group, which could not lose excited energy leading to deactivation. In this case, alternatively, the nitro group acts as an electron-acceptor to favor the fluorescence. However, the nitro-groups of B_1, B_2 or 2 tend to consume too much excited energy and act as a pure fluorescence quenchers.

The fluorescent behaviour of nitro-fluorophores in cells

The fluorescences of these compounds in hypoxic or oxic cells were also investigated. Samples from hypoxic and aerobic cell suspensions incubated with drugs for various periods of time were initially evaluated by fluorescence microscopy using appropriate excitation wavelengths. Thus a study of the time courses of accumulation of fluorescent metabolites in V79 379A Chinese hamster cells incubated with 10^{-4} M of A series and B series at 37 °C was carried out using fluorescence microscopy (Fig. 6). Further quantitative evaluation on the fluorescent intensity was performed by a fluorescence microplate reader (Fig. 4).

Figure 4 shows the time course of fluorescence development in V79 379A Chinese hamster cells incubated with compounds under hypoxic and oxic conditions. A large difference can be seen between the fluorescence of cells incubated under hypoxic and oxic conditions. After 4.5 h of exposure a 14-fold fluorescence enhancement under oxic conditions compared to hypoxic conditions could be noted for A_2 in V79 cells but for B_1 in V79 cells 12-fold fluorescence enhancement were in hypoxic cells than in oxic cells (the data for A_1 and B_2 was not shown) .

A_2 shows larger differences in fluorescence than other compounds (Fig. 5), which owes to the introduction of the methylpiperazine moiety improving the hydrophilicity and solubility in cell culture medium. For B_2 with no substituent on the naphthalene ring, the poor solubility in water is a disadvantage concerning cellular uptake. The hypoxic–oxic fluorescence ratio at exposure to B_1 could reach 12 after an incubation period of 6 h. Probably the nitro-group in naphthalene ring was reduced prior to the one in the benzene ring, which met the criterion of ICT state to give strong fluorescence.

Fluorescence microphotographs of V79 cells incubated with 10^{-4} M of these two series of hypoxic sensors were prepared (Fig. 6). Both these two series produced good accumulation and showed high differential fluorescence between hypoxic and oxic cells (V79 cells; the data for A_1 and B_2 was not shown).

Conclusion

In summary, by nitro-substituents at different positions on the aromatic rings, two series of novel reductively activated nitroheterocycles have been synthesized and evaluated as fluorescence quenching or enhancing sensors for hypoxic cells. They could be used in imaging of hypoxic cells for the first time. Depending on the substitution position and vibration amplitude, the nitro-group displays different roles. In some cases it might mainly act as normal fluorescent quencher, and sometimes mainly act as an electron-withdrawing group. An explanation for the strong fluorescence of some nitro-heterocycles could be given by infrared spectra: the restriction of the nitro-group vibration will

Table 2 Spectral data of compounds A series and B series[a,b,c]

Compound	A_{AS}/A_0	A_S/A_0
A_1	0.48	0.77
B_1	1.95	2.94
B_2	0.96	1.82
2	0.82	1.66

[a] A_{AS} The peak area of the anti-stretching vibration of the nitro-group
[b] A_S The peak area of the stretching vibration of the nitro-group
[c] A_0 The peak area of the stretching vibration of the C=O group of the corresponding compounds

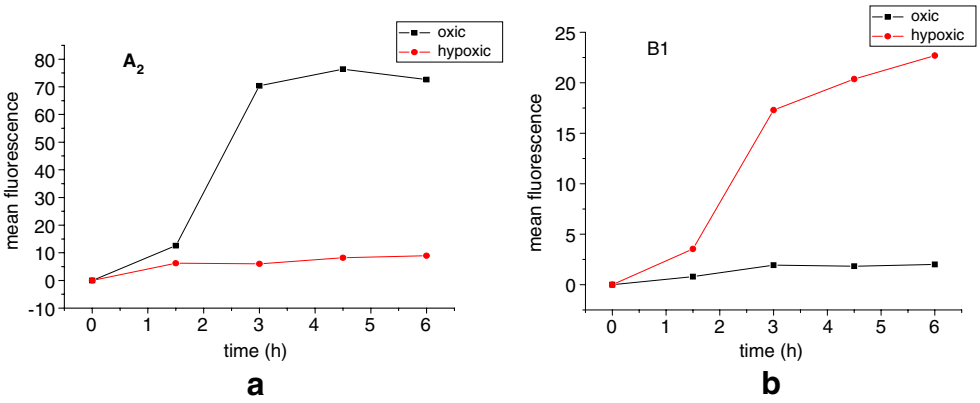

Fig. 4 The time courses of accumulation of fluorescent metabolites in V79 379A Chinese hamster cells incubated with 10^{-4} M compounds at 37 °C. **a** A_2; **b** B_1

favor its fluorescence. This might provide a novel strategy for the design of fluorescence sensors with nitro groups. Both A and B series showed very high differential fluorescence between hypoxic and oxic cells (V79 cells) in vitro. After 4.5 h the oxic–hypoxic fluorescence ratio of V79 cells incubated with A_2 reached 14, which revealed A_2 could be a promising candidate sensor for hypoxic cells and is suitable for further evaluation for hypoxic cells in tumors in vivo.

Experimental

General

All the solvents were of analytic grade. ^{1}HNMR was measured on a Bruker AV-500/400 spectrometer with chemical shifts reported as parts per million (in acetone-d_6/DMSO-d_6/$CDCl_3$-d, TMS as an internal standard). Mass spectra were measured on a HP 1100 LC-MS spectrometer. Melting points were determined with an X-6 micro-melting point apparatus and are uncorrected. Absorption spectra were determined on a PGENERAL TU-1901 UV-vis spectrometer.

Chemistry

3-Amino-10-nitro-7H-benzimidazole[2,1-a]benz[de] isoquinolin-7-one (A_1)

To 4-aminonaphthalic-1,8-anhydride 2.21 g(10 mmol) in hot glacial acetic acid (60 ml), 4-nitro-*o*-phenylenediamine 1.25 g(11 mmol) was added and the mixture was refluxed for 12 h and then cooled. The product was purified by silica column. Yield: 30%. m.p.>300 °C. ^{1} HNMR (500 MHz, Acetone-d_6), δ(ppm), 8.67 (d, 1H, J=6.23 Hz), 8.64 (d, 1H, J=7.43 Hz), 8.56 (d, 1H, J=7.29 Hz), 8.45 (d, 1H, J=7.87 Hz), 8.23 (d, 1H, J=7.87 Hz), 8.03 (t, H, J_1=7.31 Hz, J_2=7.30 Hz), 7.94 (dd, 1H, J_1=2.50 Hz, J_2=2.51 Hz), 7.39 (d, 1H, J=8.24 Hz). HRMS: $C_{18}H_{11}N_3$ calculated: 331.0831; found: 331.0830. IR(KBr), cm^{-1}: 3,300, 3,200, 3,070, 2,950, 1,685, 1,550.

3-Bromo-10-nitro-7H-Benzimidazole[2,1-a]benz[de] isoquinolin-7-one (3)

4-nitro-o-phenylenediamine 1.36 g (11 mol) was similarly condensed with 4-bromonaphthalic-1,8-anhydride 2.15 g (10 mmol) in hot glacial acetic acid under reflux conditions (for 0.5 hrs) The product was crystallized from pyridine to give pure compound. Yield: 90%. m.p.>300 °C. MS-EI: $[M]^+$ (393.1 m/z).

3-(4-Methyl-piperazin-1-yl)-10-nitro-7H-Benzimidazole [2,1-a]benz[de]isoquinolin-7-one (A_2)

A mixture of *N*-methylpiperazine (0.5 ml) and 3 (0.5 g) was refluxed in pyridine for 3 h. The crude mixture was purified

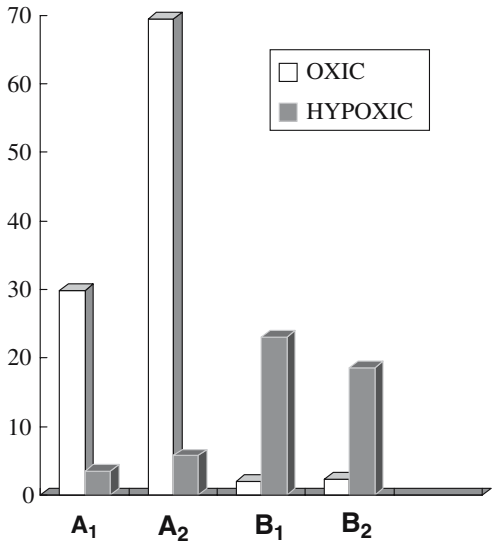

Fig. 5 Mean fluorescence intensity of V79 cells incubated with 10^{-4} M of A_1, A_2, B_1, B_2 after 4.5 h incubation at 37 °C evaluated by Fluorescence Microplate Reader

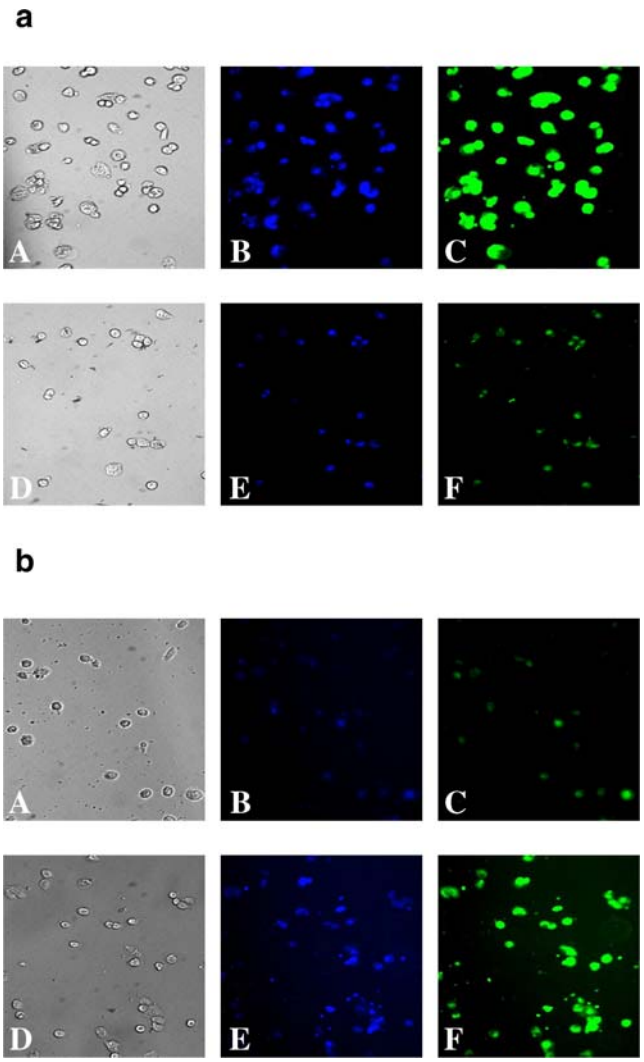

Fig. 6 Fluorescence microphotographs of V79 cells incubated with 10^{-4} M of A_2, B_1 at 37 °C. After 4.5 h incubation, scanning was taken. Magnification was ×1,000. **A** Scanning was taken on brightfield, cells on oxic condition (incubated in air and 5% CO_2); **B** excited at 359 nm, cells on oxic condition; **C** excited at 410 nm, cells on oxic condition; **D** scanning was taken on brightfield, cells on hypoxic condition (incubated in nitrogen and 5% CO_2); **E** excited at 359 nm, cells on hypoxic condition; **F** excited at 410 nm, cells on hypoxic condition. **a** Compound A_2 **b** Compound B_1

by basic Al_2O_3 column using acetone: chloroform (*v*/*v*) 1:2 as eluant to yield A_2 0.25 g. m.p.>300 °C. ^{1}HNMR (500 MHz, Acetone-d_6), δ(ppm), 8.56 (d, 1H, J= 2.29 Hz), 8.44 (d, 1H, J=7.87 Hz), 8.23 (d, 1H, J= 7.87 Hz), 8.04 (d, 1H, J=7.31 Hz), 8.02 (d, 1H, J=7.30 Hz), 7.94 (dd, 1H, J_1=2.30 Hz, J_2=2.51 Hz), 7.60 (d, 2H, J=7.82), 7.29 (d, 1H, J=8.24 Hz), 4.15 (t, 4H, J_1=4.21 Hz, J_2=4.20 Hz, –N($CH_2$$CH_2$)$_2NCH_3$), 3.64 (t, 4H, J_1=5.43 Hz, J_2=5.40 Hz, –N(CH$_2$$CH_2$)$_2NCH_3$), 3.09 (s, 3H, N(CH$_2CH_2$)$_2NCH_3$). HRMS: $C_{23}H_{19}N_5O_3$ calculated: 413.1488; found: 413.1488. IR(KBr), cm^{-1}: 3,300, 3,060, 2,965, 1,685, 1,550.

3,10-Dinitro-7H-Benzimidazole[2,1-a]benz[de]isoquinolin-7-one (B_1)

4-nitronaphthalic-1,8-anhydride 2.44 g (10 mmol) were reacted as described above with 4-nitro-*o*- phenylenediamine 1.25 g (11 mmol) to give a brown yellow solid (2.65 g). m.p. 259–261 °C. ^{1}HNMR (500 MHz, Acetone-d_6), δ(ppm), 8.55 (d, 1H, J=2.33 Hz), 8.17 (d, 1H, J= 8.15 Hz), 8.08 (d, 1H, J=6.98 Hz), 7.94 (d, 1H, J= 6.84 Hz), 7.64 (d, 1H, J=8.47 Hz), 7.50(t, 1H, J_1=7.25 Hz, J_2=7.30 Hz), 7.40 (d, 1H, J=8.23 Hz), 7.13 (d, 1H, J= 7.15 Hz). HRMS: $C_{18}H_8N_4O_5$ calculated: 360.0495; found: 360.0496. IR(KBr), cm^{-1}: 3,300, 3,070, 2,950, 1,680, 1,550.

3-Nitro-7H-Benzimidazole[2,1-a]benz[de]isoquinolin-7-one (B_2)

Similar reaction using o- phenylenediamine in glacial acetic acid gave brown yellow solid which was crystallized from toluene to yield B_2. m.p.>300 °C. ^{1}HNMR (500 MHz, DMSO-d_6), δ(ppm), 8.78 (d, 1H, J=7.2 Hz), 8.65 (d, 1H, J=8.0 Hz), 8.55 (d, 1H, J=7.8 Hz), 8.28 (d, 1H, J=8.4 Hz), 7.87 (d, 1H, J=7.8 Hz), 7.71 (t, 1H, J_1=7.8 Hz, J_2= 7.9 Hz), 7.46–7.44 (m, 2H), 7.25–7.22(m, 2H). HRMS: $C_{18}H_9N_3O_3$ calculated: 315.0644; found: 315.0644. IR (KBr), cm^{-1}: 3,300, 3,075, 2,930, 1,680, 1,550.

Biology

Compounds were initially dissolved at 1×10^{-2} M in dimethyl sulfoxide (DMSO), and small volumes were added to cell suspensions to give the appropriate concentration. The final concentration of DMSO was 1% or less. 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 cultures with 7.5% foetal calf serum. The compounds were added to cell suspensions to give the appropriate drug concentration. Then the suspension was incubated in special gases (air+5% CO_2, nitrogen+5%CO_2) at 37 °C. After various periods of incubation with these compounds, cell samples were removed centrifuged and washed with PBS to remove residual compound, resuspended in a small volume of PBS, and evaluated by Fluorescence Microscopy and Fluorescence Microplate Reader using appropriate excitation wavelength. Fluorescence microphotographs were carried out by the fluorescent microscopy of Leica Co. Lit. (DMIR 6800). The quantitative analyses was carried out by Fluorescence Microplate Reader of Leica Co. Lit.

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