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Novel nitroheterocyclic hypoxic markers for solid tumor: Synthesis and biological evaluation

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Abstract—Based on the principle that the nitro-group can quench the fluorescence and can be reduced under hypoxic conditions, several novel nitroheterocyclic compounds without 2-nitroimidazole as potential hypoxic markers were prepared. Although they were synthesized from the same matrix, nitrosubstituted acenaphtho[1,2-*b*]quinoxaline, these compounds exhibited quite different fluorescence changes when they were differently nitrosubstituted. Their evaluation for imaging tumor hypoxia was carried out in V79 cells in vitro by Fluorescence Microplate Reader. After 3.5 h, the hypoxic–oxic fluorescence differential incubated with **A1**, **A4**, and **A5** in V79 cells could reach 6, 9, and 11 times differential fluorescence between oxic and hypoxic cells separately, which are suitable for further evaluation as probes for hypoxic cells in tumors in vivo.

1. Introduction

The tumor microenvironment plays a critical role in malignant tumor progression and treatment. One of the few consistently different characteristics of solid tumor tissue compared with normal tissue is the existence in the former of significant areas which are either transiently or chronically hypoxic.[1] Hypoxic cells represent a therapeutic challenge in that these cells are refractory to radiation therapy and resistant to many of the cytotoxic drugs used in chemotherapy.[2] These hypoxic cells, however, can provide a tumor-specific targeting strategy for therapy for that the low levels of oxygen are unique features of solid tumors and under normal physiological conditions they do not occur in normal tissues. Subsequently, there is increasing interest in the detection of hypoxic cell fractions in tumors so that optimal treatment schedule could be devised for individual patients. To measure tumor oxygenation in experimental or clinical tumors, different techniques have been used by various methods, several are based on the hypoxia-dependent bioreduction metabolism of a labeled 2-nitroimidazole. Various labels have been proposed including ^{3}H, ^{14}C, ^{75}Br, ^{76}Br, ^{77}Br, and ^{19}F, NMR, PET, autoradiography, and fluorescence immunohistochemistry have been used to detect bioreductively bound metabolites of labeled nitroimidazoles.[3–7] An alternative approach is to use the metabolic binding of fluorescent nitroaromatic compounds. The advantage of this approach is that not only can the fluorescence be visualized in histological section, but it lends itself to quantitation (including cell population distributions) using flow cytometry or other instruments.[8–10]

The chemical basis of the selective labeling of hypoxic cells is that these cells are known to reduce the nitro-group to the amine-group, a process preceded by an initial one-electron reduction step that is reversed in oxic cells but not in hypoxic cells.[11] Numerous nitroaromatic structures have been evaluated in model system in vitro, but many were increased fluorescent markers for hypoxia with the principle that the nitro-group quenches the fluorescence of the aromatic ring system, but on bioreduction of the nitro-group in hypoxic cells the ring system becomes fluorescent.[12] And until recently, although a number of types of nitroaromatic compounds have shown promise in identifying hypoxic cells in tumors, many of these compounds have been based on the expensive 2-nitroimidazole. Therefore, we hope to envisage a potential probe which has distinguished fluorescent difference between hypoxic cells and oxic cells from the easily synthesized compounds containing the active group in low cost instead of 2-nitroimidazole. At first, a suitable fluorophore is required, which should

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have an ideal excitation wavelength, a high quantum efficiency, and an excellent fluorescence difference between hypoxic and oxic cells. Certain naphthalimides and their derivatives have been reported to have absorption at 350 nm and fluorescence emission between 450 and 550 nm. The fluorescence quantum yields are also good. And a number of novel nitronaphthalimides have been reported as potential fluorescent probes for hypoxic cells in tumors already.[13] Recently, several researchers have modified these compounds to exploit increased fluorescent markers for hypoxic cells, relying on the quenching of fluorescence by the nitro-group to produce weak fluorescence in oxic cells and the bioreduction of the nitro-group in hypoxic cells to give a strong differential fluorescence.[14] In this paper, we report the synthesis and evaluation of the hypoxic probes based on nitrosubstituted acenaphtho[1,2-*b*]quinoxaline, considering the following reasons: on one hand, acenaphtho[1,2-*b*]quinoxaline has been widely used as one kind of antitumor drug, with the nitro-group, it can bind with macromolecules in the tumor cells more efficiently.[15] And on the other hand, 6-aminoquinoxaline has been widely used as a kind of fluorophore since 1970s, whose yellow fluorescence can be easily distinguished from the weak blue fluorescence of the tumor cells.

2. Results and discussion

2.1. Synthesis and spectra data

2.1.1. The synthetic route is shown in Figure 1. The nitrosubstituted acenaphtho[1,2-*b*]quinoxalines were prepared from 3-substituted acenaphthene quinine and ortho-diamines as shown in Figure 1. To 3-nitroacenaphthene quinone (1 mmol) in hot glacial acetic (20 ml), 4-nitro-*o*-phenylenediamine (1.3 mmol) was added and the mixture was refluxed for 1 h and then allowed to cool. An orange solid was precipitated and filtered, and crystallized in DMF/H_2O to yield the desired compound **A1**. Compounds **A2**, **A3**, **A6**, and **B** were obtained as described for **A1**. The 3-(4-methyl-piperazin-1-yl)-9-nitro-acenaphtho [1,2-*b*]quinoxaline (**A4**) and *N*,*N*-dimethyl-*N*′-(7-nitro-benzo[1,2,5]oxadiazole-4-yl)-ethane-1,2-diamine (**A5**) were obtained by refluxing ammonia in DMF to get corresponding substituents. In order to compare the fluorescence differential between the nitrosubstituted acenaphtho[1,2-*b*]quinoxalines and the aminosubstituted acenaphtho[1,2-*b*]quinoxalines, **A9**, **A10**, and **A11** were synthesized from **A1**, **A2**, and **A3**. It has been reported that when the absorption wavelength <500 nm, the nitro-group is an efficient fluorescence quencher.[16–18] As described in Table 1 and Figure 2, 6-aminoquinoxaline is a good fluorophore with quantum value $\Phi = 0.165$. Interestingly, the compound **A11** showed $\Phi = 0.047$, but another different aminosubstituted **A10** gave much lower quantum yield (<0.0003). It is possible that in the acenaphtho[1,2-*b*]quinoxaline ring system, the amino-group in phenazine ring plays more important role as electron-donating group in fluorescence than the one in naphthalene ring, and 3-substituent of the ring system has little influence over the fluorescence so that **A9** and **A6** still showed good fluorescence no matter whether nitro substituted or amino substituted. Based on this phenomenon, it is worthy noting that the nitro-group in quinoxaline-ring here is a pure fluorescence quencher, which implies that integra-

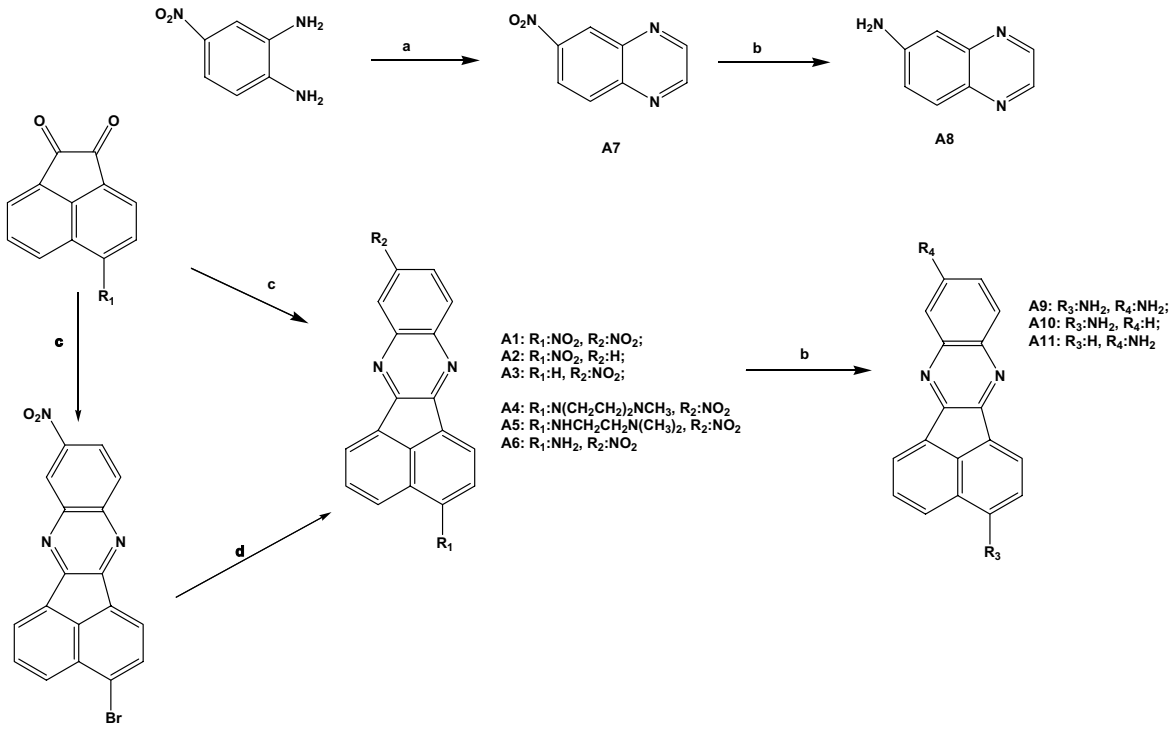

Figure 1. Synthesis of target compounds. Reagents and conditions: (a) acetonitrile, 50 °C; (b) $SnCl_2$/HCl, 80 °C; (c) acetic acid, refluxing; (d) DMF, refluxing.

Table 1. Spectra data of compounds[a,b]

Compound	UV λ_{max} (nm) (lg ε)	FL λ_{max} (nm) (Φ)
A1	324 (4.70)	None
A2	328 (4.32)	None
A3	348 (4.46)	None
A4	437 (4.23)	None
A5	324 (4.85)	None
A6	390 (4.39)	557 (0.025)
A7	254 (3.99)	None
A8	392 (3.88)	514 (0.165)
A9	361 (3.47)	526 (0.038)
A10	412 (4.27)	None
A11	314 (4.28)	556 (0.047)

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

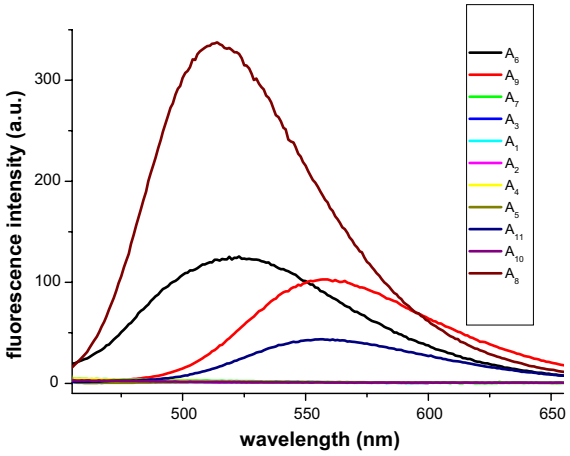

Figure 2. Fluorescence intensity of compounds **A1**–**A11** when concentration of compound was 10^{-5} mol/L in absolute methanol solution.

tion of acenaphtho[1,2-*b*]quinoxaline might not impair the fluorescence properties of the aminophenazine fluorophore, and it is the phenazine-localized orbital that acts as a fluorophore.[19] It was expected that these series of compounds were expected to show large fluorescence differential between hypoxic and oxic cells when the nitro-group was reduced to amino-group.

2.2. Biological studies

A study of the time courses of accumulation of fluorescent metabolites in V79 379A Chinese hamster cells incubated with 10^{-4} M of reduced and increased fluorescent markers for hypoxic cells at 37 °C separately was carried out with fluorescence microscopy. Further quantitative evaluation of the fluorescent intensity was carried out by Fluorescence Microplate Reader.

By fluorescence microscopy, we got the fluorescence microphotographs of V79 cells incubated with 10^{-4} M of these two series of hypoxic markers. Figure 3 gives the evaluation of acenaphtho[1,2-*b*]quinoxaline derivatives in hypoxic and oxic cells. As typical fluorescence-quenching group, the hypoxia-dependent bioreductive metabolism of the nitro-group in benzene ring made significant differential between the fluorescence of cells

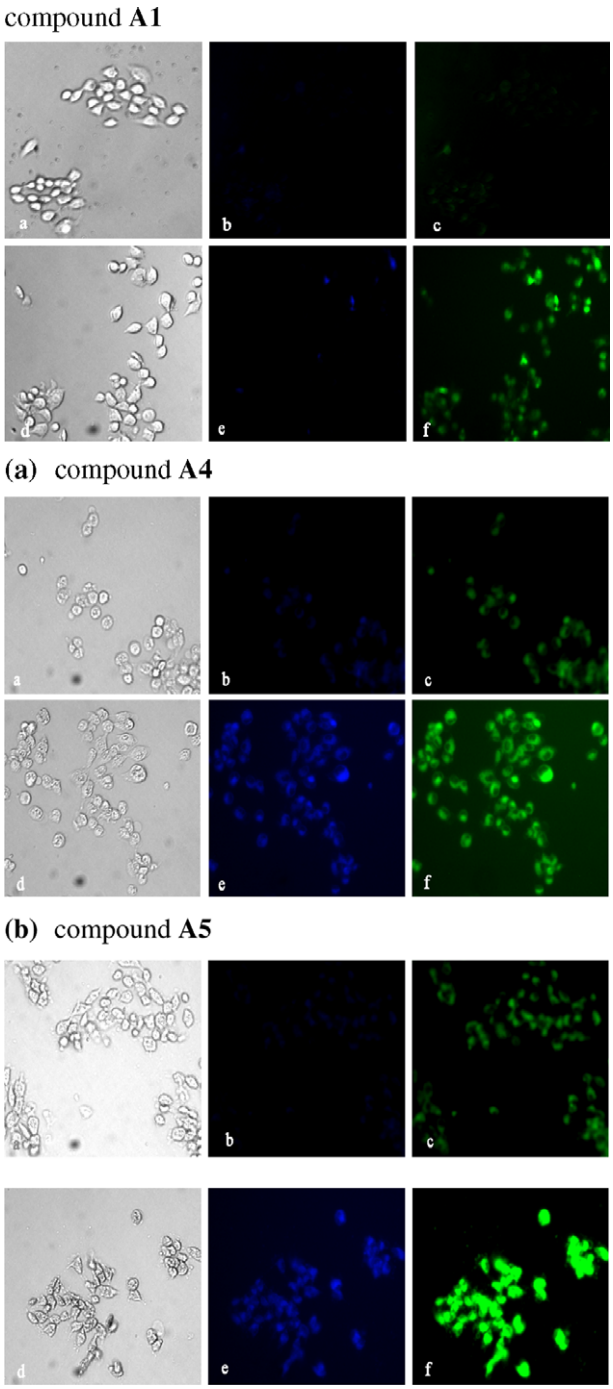

Figure 3. Fluorescence microphotographs of V79 cells incubated with **A1**, **A4**, and **A5** at 37 °C. After 3.5 h incubation, scanning was taken. Magnification is 1000×. (a) Scanning was taken on brightfield, cells on oxic condition (incubated in air and 5% CO_2); (b) excited at 359 nm, cells on oxic 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.

incubated under oxic and hypoxic conditions when the nitro-group was reduced to amino-group. The hypoxic–oxic fluorescence differential could reach 6, 9, and 11 times when incubated with **A1**, **A4**, and **A5** in V79 cells for 3.5 h (Fig. 4). But the big problem for these compounds is their water-solubility, **A2** and **A3** were

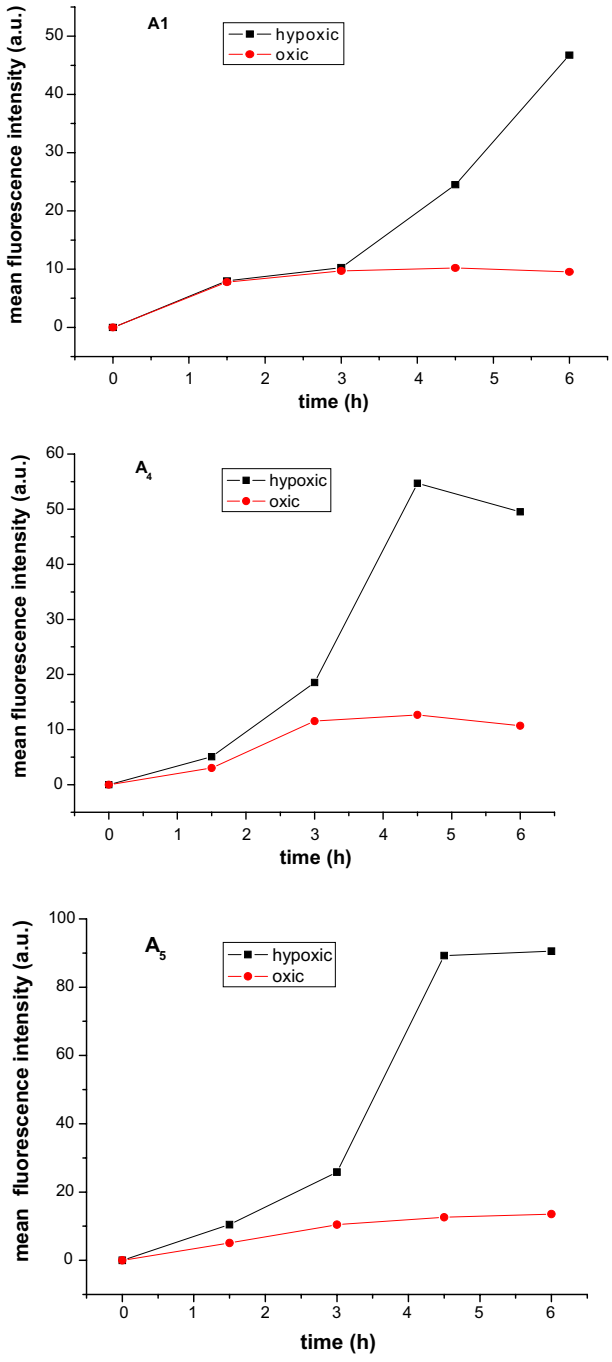

Figure 4. The time courses of accumulation of fluorescent metabolites in V79 Chinese cells incubated with 10^{-4} M of (a) **A1**, (b) **A4**, and (c) **A5**.

insoluble in culture medium and deposited outside the cells, which made the quantitative analysis impossible. The compound **A5** with *N*,*N*-dimethyl aminoethyl group had the highest hypoxic–oxic ratio. One of the reasons was that the nitrogen atom of *N*,*N*-dimethyl aminoethyl group was easily protonated in aqueous solution at the physiological pH, in this case, the water-solubility was improved.[20] The other was that compound **A5** with the *N*,*N*-dimethyl group had good binding ability with the double strand DNA, so the cell-uptake of this compound would be increased and the speed of metabolism was decreased.[21]

3. Conclusion

A novel series of reductively activated nitroheterocyclic compounds have been prepared. We have demonstrated that this active group was synthesized easily from cheap raw materials without basing on inducting expensive 2-nitroimidazole directly. Surprisingly small difference between the structures of the fluorescent rings can lead to large differences in overall cellular fluorescence, for example, though based on the same matrix nitrosubstituted acenaphtho[1,2-*b*]quinoxaline, by nitro-group substituted in different sites, several hypoxic markers have been synthesized and evaluated: reduced fluorescent markers for hypoxic cells and increased fluorescent markers for hypoxic cells. They all show very large differential fluorescence between hypoxic and oxic cells (V79 cells) in vitro. After 3.5 h, the hypoxic–oxic fluorescence differential incubated with **A1**, **A4**, and **A5** in V79 cells could reach 6, 9, and 11 times differential fluorescence between oxic and hypoxic cells separately, which are suitable for further evaluation as probes for hypoxic cells in tumors in vivo.

4. Experimental

4.1. Materials and methods

All the solvents were of analytic grade. ^{1}H NMR 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 point were determined with an X-6micro-melting point apparatus and are uncorrected. Absorption spectra were determined on a Cary 100 UV–vis spectrometer and fluorescence spectra were determined on a Cary Eclipse fluorescence spectrometer.

4.2. Synthesis

4.2.1. Synthesis of 3,9-dinitro-acenaphtho[1,2-*b*]quinoxaline (A1). To 3-nitroacenaphthene quinone (1 mmol) in hot glacial acetic acid (20 ml), 4-nitro-*o*-phenylenediamine (1.3 mmol) was added and the mixture was refluxed for 1 h and then allowed to cool. An orange solid was precipitated and filtered, and crystallized in DMF/H_2O to yield the desired compound **A1**. Yield 80%. Mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.00 (d, 1H, J = 2.40 Hz), 8.57–8.52 (m, 3H), 8.44 (d, 1H, J = 6.40 Hz), 8.42 (d, 1H, J = 3.20 Hz), 8.03 (d, 1H, J = 8.00 Hz), 8.00 (d, 1H, J = 7.20 Hz). HRMS: $C_{18}H_8N_4O_4$ calculated: 344.0546. Found: 344.0542. IR (KBr), cm^{-1}: 3452, 1588, 1527, 1341, 1070.

4.2.2. Synthesis of 3-nitro-acenaphtho[1,2-*b*]quinoxaline (A2). The compound was prepared on a 0.1 mmol scale from 3-nitroacenaphthene quinone and *o*-phenylenediamine as described for **A1** and crystallized in DMF/H_2O to yield the desired compound **A2**. Yield 85%. Mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.82 (d, 1H, J = 7.60 Hz), 8.75 (d, 1H, J = 8.80 Hz), 8.57 (d, 1H, J = 8.00 Hz), 8.54 (d, 1H, J = 7.60 Hz), 8.27–

8.23 (m, 2H), 8.17 (t, 1H, J_1 = 8.00 Hz, J_2 = 8.00 Hz), 7.93–7.90 (m, 2H). HRMS: $C_{18}H_9N_3O_2$ calculated: 299.0695. Found: 299.0696. IR (KBr), cm^{-1}: 3407, 2919, 1521, 1315, 1190, 1085.

4.2.3. Synthesis of 9-nitro-acenaphtho[1,2-*b*]quinoxaline (A3). The compound was prepared on a 0.1 mmol scale from acenaphthene quinone and 4-nitro-*o*-phenylenediamine as described for **A1** and crystallized in DMF/H_2O to yield the desired compound **A3**. Yield 80%. Mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm), 8.99 (d, 1H, J = 2.40 Hz), 8.57–8.52 (m, 3H), 8.44–8.39 (m, 3H), 8.02 (t, 2H, J_1 = 8.00 Hz, J_2 = 7.20 Hz). HRMS: $C_{18}H_9N_3O_2$ calculated: 299.0695. Found: 299.0694. IR (KBr), cm^{-1}: 3072, 2919, 1526, 1339, 1071.

4.2.4. Synthesis of 3-nitro-acenaphtho[1,2-*b*]quinoxaline-9-ylamine (A6). The compound was prepared on a 0.1 mmol scale from 3-aminoacenaphthene quinone and 4-amino-*o*-phenylenediamine as described for **A1** and crystallized in DMF/H_2O to yield the desired compound **A6**. Yield 80%. Mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm), 8.28 (d, 1H, J = 1.2 Hz), 8.21 (d, 1H, J = 8.80 Hz), 8.16 (d, 1H, J = 8.40 Hz), 7.92–7.89 (m, 2H), 7.34–7.29 (m, 2H). MS-EI: $[M]^+$ (269.0 *m*/*z*). IR (KBr), cm^{-1}: 3407, 2919, 1631, 1578, 1382, 1099.

4.2.5. Synthesis of 3-bromo-9-nitro-acenaphtho[1,2-*b*]quinoxaline (B). The compound was prepared on a 0.1 mmol scale from 3-bromoacenaphthene quinone and 4-nitro-*o*-phenylenediamine as described for **A1** and crystallized in DMF/H_2O to yield the desired compound **B**. Yield 80%. MS-EI: $[M]^+$ (377.0 *m*/*z*).

4.2.6. Synthesis of 3-(4-methyl-piperazin-1-yl)-9-nitro-acenaphtho[1,2-*b*]quinoxaline (A4). To 4-methyl-piperazine 2.21 g (10 mmol) in hot DMF (60 ml), 3-bromo-9-nitro-acenaphtho[1,2-*b*]quinoxaline 1.25 g (11 mmol) was added and the mixture was refluxed for 5 h and then allowed to cool in water. A brown solid was precipitated and filtered, and chromatographed over silica gel column using CH_2Cl_2/methanol (v/v) 4:1 as eluent to yield the desired compound A4. Yield 50%. Mp > 300 °C. ^{1}H NMR (400 MHz, acetone-d_6), δ (ppm), 8.95 (d, 1H, J = 2.40 Hz), 8.56–8.50 (m, 2H), 8.46 (d, 1H, J = 8.40 Hz), 8.41 (d, 1H, J = 8.00 Hz), 8.38 (d, 1H, J = 9.20 Hz), 7.96 (t, 1H, J_1 = 7.80 Hz, J_2 = 7.80 Hz), 7.44 (d, 1H, J = 8.00 Hz), 3.41 (s, 4H, –N(CH_2CH_2)$_2$NCH$_3$), 2.77 (s, 4H, –N(CH$_2$$CH_2$)$_2NCH_3$), 2.41 (s, 3H, –N(CH$_2CH_2$)$_2$ NCH_3). HRMS: $C_{23}H_{19}N_5O_2$ calculated: 397.1539. Found: 397.1539. IR (KBr), cm^{-1}: 3436, 2947, 1612, 1492, 1432, 1349, 1229, 1006.

4.2.7. Synthesis of *N*,*N*-dimethyl-*N*′-(9-nitro-acenaphtho[1,2-*b*]quinoxalin-3-yl)-ethane-1,2-diamine (A5). The compound was prepared on a 0.1 mmol scale from 3-bromo-9-nitro-acenaphtho[1,2-*b*]quinoxaline and *N*,*N*-dimethyl-ethane-1,2-diamine as described for **A4** to yield the desired compound **A5**. Yield 40%. Mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm), 8.85 (dd, 1H, J_1 = 2.40 Hz, J_2 = 2.40 Hz, J_3 = 24.00 Hz), 8.62 (m, 1H), 8.48–8.38 (m, 2H), 8.29 (t, 1H, J_1 = 10.00 Hz, J_2 = 9.20 Hz), 8.22 (t, 1H, J_1 = 9.60 Hz, J_2 = 9.20 Hz), 7.84 (t, 1H, J_1 = 8.40 Hz, J_2 = 7.20 Hz), 6.88 (t, 1H, J_1 = 6.40 Hz, J_2 = 8.00 Hz), 3.56 (t, 2H, J_1 = 3.20 Hz, J_2 = 6.40 Hz, –NCH_2CH$_2$N(CH$_3$)$_2$), 2.37 (s, 2H, –NCH$_2$$CH_2$N(CH$_3$)$_2$), 2.77 (s, 6H, –NCH$_2CH_2$N($CH_3$)$_2$). HRMS: $C_{22}H_{19}N_5O_2$ calculated: 385.1539. Found: 385.1551. IR (KBr), cm^{-1}: 3339, 2910, 1602, 1521, 1339, 1071.

4.2.8. Synthesis of 6-nitroquinoxaline (A7). To 40% oxalaldehyde (5 ml) in MeCN (50 ml) was added 4-nitro-*o*-phenylenediamine (4.0 g) at 50 °C slowly. The mixture was kept stirring for 15 h and cooled down, diluted with H_2O (150 ml) to afford brown precipitate, which was chromatographed over silica gel column using ethylacetate/petroleum ether (v/v) 5:1 as eluent to yield the desired compound **A7**. Yield 85%. Mp 174 °C. ^{1}H NMR (400 MHz, acetone-d_6), δ (ppm), 9.15 (s, 2H, –NCH=CHN), 8.95 (d, 1H, J = 2.80 Hz), 8.64–8.61 (M, 1H), 8.37 (t, 1H, J_1 = 4.00 Hz, J_2 = 8.80 Hz). HRMS: $C_8H_5N_3O_2$ calculated: 175.0382. Found: 175.0378. IR (KBr), cm^{-1}: 3368, 3053, 1521, 1344, 1071, 1023.

4.2.9. Synthesis of 6-aminoquinoxaline (A8). The compound was prepared on a 0.1 mmol scale from **A7**. To compound **A7** 3.30 g (10 mmol) in concentrated hydrochloric acid (25 ml), $SnCl_2$ 40 mmol was added and the mixture was stirred for 4 h at 80 °C and then allowed to cool. The brown solid was precipitated and filtered, and chromatographed over silica gel column using ethylacetate/petroleum ether (v/v) 2:1 as eluent to yield the desired compound **A8**. Yield 67%. Mp 157 °C. ^{1}H NMR (400 MHz, acetone-d_6), δ (ppm), 8.60 (d, 1H, J = 2.00 Hz), 8.48 (d, 1H, J = 2.00 Hz), 7.77 (d, 1H, J = 9.2 Hz), 7.35–7.32 (m, 1H), 7.09 (d, 1H, J = 2.4 Hz), 5.53 (s, 2H, –NH_2). HRMS: $C_8H_7N_3$ calculated: 145.0640. Found: 145.0631. IR (KBr), cm^{-1}: 3395, 3310, 3190, 1648, 1616, 1505, 1310, 1032.

4.2.10. Synthesis of acenaphtho[1,2-*b*]quinoxaline-3,9-diamine (A9). The compound was prepared on a 0.1 mmol scale from **A1** as described for **A8** to yield the desired compound **A9**. Yield 40%. Mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm), 8.36 (d, 1H, J = 8.40 Hz), 8.25 (d, 1H, J = 6.80 Hz), 8.09 (d, 1H, J = 7.60 Hz), 7.84 (d, 1H, J = 9.2 Hz), 7.74 (t, 1H, J_1 = 7.20 Hz, J_2 = 8.00 Hz), 7.21–7.19 (m, 2H), 7.00 (d, 1H, J = 8.00 Hz). MS-EI: $[M]^+$ (284.1 *m*/*z*). IR (KBr), cm^{-1}: 3311, 2919, 1631, 1574, 1377, 1099.

4.2.11. Synthesis of acenaphtho[1,2-*b*]quinoxaline-3-ylamine (A10). The compound was prepared on a 0.1 mmol scale from **A2** as described for **A8** to yield the desired compound **A10**. Yield 50%. Mp > 300 °C. ^{1}H NMR (400 MHz, acetone-d_6), δ (ppm), 8.49 (d, 1H, J = 8.40 Hz), 8.17 (d, 1H, J = 6.80 Hz), 7.83 (t, 1H, J_1 = 7.60 Hz, J_2 = 8.00 Hz), 7.79–7.71 (m, 2H), 7.07 (d, 1H, J = 8.00 Hz), 6.51 (s, 2H, –NH_2). HRMS: $C_{18}H_{11}N_3$ calculated: 269.0953. Found: 269.0968. IR (KBr), cm^{-1}: 3445, 3148, 2919, 1598, 1406, 1272, 1085.

4.2.12. Synthesis of acenaphtho[1,2-*b*]quinoxaline-9-ylamine (A11). The compound was prepared on a 0.1 mmol scale from **A3** as described for **A8** to yield the desired compound **A11**. Yield 50%. Mp > 300 °C. ^{1}H NMR

(400 MHz, acetone-d_6), δ (ppm), 8.99 (d, 1H, $J = 2.40$ Hz), 8.57–8.52 (m, 3H), 8.44–8.39 (m, 3H), 8.02 (t, 2H, 1H, $J_1 = 8.00$ Hz, $J_2 = 7.20$ Hz). HRMS: $C_{18}H_{11}N_3$ calculated: 269.0953. Found: 269.0954. IR (KBr), cm^{-1}: 3321, 2928, 1626, 1578, 1372, 1104.

4.3. Biology

Compounds were initially dissolved at 1×10^{-2} M in dimethylsulfoxide (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% fetal 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.

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