

Supporting Information

Design, Synthesis and Evaluation of the Antiproliferative Activity of a Series of Novel Fused Xanthenone Aminoderivatives in Human Breast Cancer Cells

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General experimental procedures

All chemicals were purchased from Aldrich Chemical Co. Melting points were determined on a Büchi apparatus and are uncorrected. ^1H NMR spectra and 2D spectra were recorded on a Bruker Avance 400 instrument, whereas ^{13}C NMR spectra were recorded on a Bruker AC 200 spectrometer in deuterated solvents and were referenced to TMS (δ scale). The signals of ^1H and ^{13}C spectra were unambiguously assigned by using 2D NMR techniques: ^1H - ^1H COSY, NOESY HMQC and HMBC. Analytical thin layer chromatography (TLC) was carried out on precoated (0.25 mm) Merck silica gel F-254 plates. Elemental analyses were performed on a Perkin-Elmer PE 240C Elemental Analyzer (Norwalk, CT) and are within $\pm 0.4\%$ of the theoretical values.

Synthesis and characterization of compounds

3,5-Dichloro-2-nitroaniline (5): To a solution of 2,4,6-trichloro-1-nitrobenzene⁹ (**1**, 10 g, 42.92 mmol) in anhydrous DMSO (30 ml) was added sodium azide (8.37 g, 128.76 mmol) and the resulting mixture was stirred at room temperature for 20 h. The reaction mixture was poured into ice/water and the resulting solid was filtered, washed with water and air-dried to yield a mixture of compounds **2** and **3**, which without further purification, was dissolved in a 1:1 mixture of CH_2Cl_2 : EtOH (10 ml). The resulting solution was added to a suspension of $\text{CuSO}_4 \cdot 2\text{H}_2\text{O}$ (0.062 g, 0.198 mmol) and sodium borohydride (0.328 g, 8.94 mmol) in ethanol (30 ml) at 0°C . To this solution was then added sodium borohydride (0.492 g, 12.22 mmol) in small portions and the reaction was stirred at room temperature for 1 h. The reaction mixture was then made alkaline with a 40% sodium hydroxide solution (pH~12),

vacuum-evaporated, extracted with EtOAc – water, the organic layer was dried (Na_2SO_4) and evaporated to dryness. Flash chromatography on silica gel using a mixture of cyclohexane:EtOAc 5:1 as the eluent provided compounds **4** and **5**.

Data for 3,5-Dichloro-4-nitroaniline (4): Yield: 8%; mp 173-174 °C (EtOH) lit.^{11a} 169-170 °C; ^1H NMR (CDCl_3 , 400 MHz) δ (ppm) 4.14 (s, 2H, D_2O exch, NH_2), 6.57 (s, 2H, H-2, H-6); ^{13}C NMR (CDCl_3 , 50 MHz) δ (ppm) 108.86 (C-1), 113.43 (C-2, C-6), 127.79 (C-3, C-5), 148.46 (C-4).

Data for 3,5-Dichloro-2-nitroaniline (5): Yield: 86%; mp 78-79 °C (EtOH) lit.^{11a} 78-79 °C; ^1H NMR (CDCl_3 , 400 MHz) δ (ppm) 5.09 (s, 2H, D_2O exch, NH_2), 6.71 (d, 1H, $J = 2$ Hz, H-6), 6.79 (d, 1H, $J = 2$ Hz, H-4); ^{13}C NMR (CDCl_3 , 50 MHz) δ (ppm) 115.89 (C-6), 120.06 (C-4), 129.71 (C-3), 133.17 (C-2), 138.49 (C-5), 143.63 (C-1).

3,5-Dichloro-2-nitroacetanilide (6). To a suspension of **5** (6.08 g, 29.37 mmol) in glacial acetic acid (10 ml) was added acetic anhydride (11 ml, 117.48 mmol) and the resulting mixture was refluxed for 3 h. Upon cooling, it was poured into ice/water and the resulting solid was filtered, washed with water and air-dried to give the nitro compound **6**. Yield 97%; mp 141-143 °C (EtOH) lit.^{11b} 138-139 °C; ^1H NMR (CDCl_3 , 400 MHz) δ (ppm) 2.21 (s, 3H, COCH_3), 7.27 (d, 1H, $J = 2$ Hz, H-4), 8.17 (s, 1H, D_2O exch., NH), 8.35 (d, 1H, $J = 2$ Hz, H-6); ^{13}C NMR (CDCl_3 , 50 MHz) δ (ppm) 24.73 (COCH_3), 121.92 (C-5), 122.13 (C-6), 126.00 (C-4), 127.83 (C-3), 132.83 (C-1), 138.25 (C-2), 168.69 (NHCO).

Ethyl 2-(3-acetylamino-5-chloro-2-nitrophenyloxy)benzoate (8) and Ethyl 2-(3-acetylamino-5-chloro-4-nitrophenyloxy)benzoate (7). To a solution of ethyl salicylate (14.18 g, 85.42 mmol) in dry pyridine (60 mL), were added under argon

3,5-dichloro-2-nitroacetanilide (**6**, 7.09 g, 28.47 mmol), powdered K_2CO_3 (11.81 g, 85.42 mmol) and CuO (2.26 g, 28.47 mmol) and the mixture was heated at reflux for 36h. Upon cooling, the reaction mixture was acidified with 36% HCl (pH~4) and the inorganic material was filtered off. The filtrate was extracted with diethylether (3x40 mL), the combined organic extracts were washed with brine, dried (Na_2SO_4) and concentrated to dryness. Flash chromatography on silica gel, using a mixture of cyclohexane:EtOAc 7:1 as the eluent, provided compounds **7** and **8**.

Data for Ethyl 2-(3-acetylamino-5-chloro-4-nitrophenyloxy)benzoate (7): Yield: 8%. Oil; 1H NMR ($CDCl_3$, 400 MHz) δ (ppm) 1.13 (t, 3H, $J= 8$ Hz, CH_2CH_3), 2.06 (s, 3H, $COCH_3$), 4.16 (q, 2H, $J= 8$ Hz, CH_2CH_3), 6.63 (d, 1H, $J= 2$ Hz, H-6'), 7.07 (dd, 1H, $J= 8$ Hz, 2 Hz, H-3), 7.26 (td, 1H, $J= 8$ Hz, 2 Hz, H-5), 7.51 (td, 1H, $J= 8$ Hz, 2 Hz, H-4), 7.61 (d, 1H, $J= 2$ Hz, H-2'), 7.95 (dd, 1H, $J= 8$ Hz, 2 Hz, H-6), 8.65 (s, 1H, D_2O exch., NH); ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm) 13.93 (CH_2CH_3), 24.42 ($COCH_3$), 61.26 (CH_2CH_3), 109.48 (C-2'), 113.70 (C-6'), 123.16 (C-3), 124.24 (C-5'), 126.08 (C-5), 128.43 (C-3'), 132.45 (C-6), 133.93 (C-1), 134.29 (C-4), 135.05 (C-4'), 152.82 (C-2), 160.70 (C-1'), 164.56 ($COCH_2CH_3$), 169.25 (NHCO). Anal. ($C_{17}H_{15}ClN_2O_6$) C, H, N.

Data for Ethyl 2-(3-acetylamino-5-chloro-2-nitrophenyloxy)benzoate (8): Yield: 47%; mp 139-141 $^{\circ}C$ (EtOAc); 1H NMR ($CDCl_3$, 400 MHz) δ (ppm) 1.21 (t, 3H, $J= 8$ Hz, CH_2CH_3), 2.26 (s, 3H, $COCH_3$), 4.27 (q, 2H, $J= 8$ Hz, CH_2CH_3), 6.42 (d, 1H, $J= 2$ Hz, H-6'), 7.20 (dd, 1H, $J= 8$ Hz, 2 Hz, H-3), 7.40 (td, 1H, $J= 8$ Hz, 2 Hz, H-5), 7.64 (td, 1H, $J= 8$ Hz, 2 Hz, H-4), 8.06 (dd, 1H, $J= 8$ Hz, 2 Hz, H-6), 8.16 (d, 1H, $J= 2$ Hz, H-4'), 8.66 (s, 1H, D_2O exch., NH); ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm) 13.96 (CH_2CH_3), 25.03 ($COCH_3$), 61.45 (CH_2CH_3), 112.13 (C-6'), 116.04 (C-4'), 122.89 (C-3), 123.40 (C-1), 124.09 (C-3'), 126.27 (C-5), 132.61 (C-6), 133.19 (C-2'), 134.34

(C-4), 139.12 (C-5'), 152.61 (C-1'), 152.80 (C-2), 164.41 (COOCH₂CH₃), 168.68 (NHCO). Anal. (C₁₇H₁₅ClN₂O₆) C, H, N.

2-(3-Acetylamino-5-chloro-2-nitrophenyloxy)benzoic acid (9). To a solution of **8** (4 g, 10.57 mmol) in dioxane at room temperature, was added dropwise a cold 40% NaOH solution (3.17 g, 31.70 mmol). The mixture was stirred for 2h at room temperature and then poured into ice/water and acidified with a 18% HCl solution (pH~5). The precipitate was filtered, washed with water and air-dried to furnish compound **9** (3.66 g, 99%); mp 209-211 °C (EtOH); ¹H-NMR (DMSO-*d*₆, 400 MHz) δ (ppm) 2.06 (s, 3H, COCH₃), 6.54 (d, 1H, *J*= 2 Hz, H-6'), 7.30 (dd, 1H, *J*= 8 Hz, 2 Hz, H-3), 7.40 (d, 1H, *J*= 2 Hz, H-4'), 7.44 (td, 1H, *J*= 8 Hz, 2 Hz, H-5), 7.70 (td, 1H, *J*= 8 Hz, 2 Hz, H-4), 7.94 (dd, 1H, *J*= 8 Hz, 2 Hz, H-6), 10.27 (s, 1H, D₂O exch., NH); ¹³C-NMR (DMSO-*d*₆, 50 Hz) δ (ppm) 23.66 (COCH₃), 112.83 (C-6'), 118.78 (C-4'), 123.02 (C-3), 124.63 (C-1), 126.79 (C-3'), 132.61 (C-5), 133.30 (C-6), 134.52 (C-2'), 134.92 (C-4), 136.18 (C-5'), 152.69 (C-1'), 152.91 (C-2), ,165.91 (COOH), 169.45 (NHCO). Anal. (C₁₅H₁₁ClN₂O₆) C, H, N.

N-(1-Chloro-4-nitro-9-oxo-9H-xanthen-3-yl)acetamide (10). Compound **9** (1 g, 2.85 mmol) was added in small portions to a mixture of acetic anhydride (5.40 mL, 57.13 mmol) and sulfuric acid 98% (10 ml) and the resulting suspension was heated at 90 °C for 50 min. Upon cooling the mixture was poured into ice-water, the precipitate was filtered and air-dried to give crude **10**, which was purified by column chromatography (silica gel) using a mixture of CH₂Cl₂:cyclohexane 1:4 as the eluent. Yield: 91%; mp >270 °C (DMF-H₂O); ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm) 2.03 (s, 3H, COCH₃), 7.43-7.52 (m, 2H, H-5, H-7), 7.81 (td, 1H, *J*= 8 Hz, 2 Hz, H-6), 7.99

(s, 1H, H-2), 8.10 (dd, 1H, $J = 8$ Hz, 2 Hz, H-8), 10.64 (s, 1H, D₂O exch, NH); ¹³C-NMR (DMSO-*d*₆, 50 Hz) δ (ppm) 25.51 (COCH₃), 111.60, 121.91, 131.18, 135.22, 140.48 (C-1, C-3, C-4, C-4a, C-9a), 117.93 (C-5), 121.49 (C-2), 122.18 (C-8a), 125.63 (C-7), 126.54 (C-8), 135.79 (C-6), 154.16 (C-10a), 172.26 (COCH₃), 173.18 (C-9). Anal. (C₁₅H₉ClN₂O₅) C, H, N.

***N*-[1-[2-(Dimethylamino)ethyl]amino-4-nitro-9-oxo-9*H*-xanthen-3-yl]acetamide**

(11a). A solution of **10** (327 mg, 0.983 mmol) and 2-dimethylaminoethylamine (1.02 mL, 9.3 mmol) in dry DMSO (6 mL) was refluxed for 1 h. Upon cooling, the mixture was poured into ice-water, extracted with EtOAc – water, the organic layer was dried (Na₂SO₄) and evaporated to dryness. The residue was purified by column chromatography (silica gel, CH₂Cl₂:MeOH 100:2) to furnish **11a** (351 mg, 93%); mp 204-206 °C (EtOAc - *n*-hexane); ¹H-NMR (CDCl₃, 400 MHz) δ (ppm) 2.26 (s, 3H, COCH₃), 2.34 (s, 6H, N(CH₃)₂), 2.66 (t, 2H, $J = 7$ Hz, NHCH₂CH₂N), 3.39 (q, 2H, $J = 7$ Hz, 5 Hz, NHCH₂CH₂N), 7.36 (t, 1H, $J = 8$ Hz, H-7), 7.44 (d, 1H, $J = 8$ Hz, H-5), 7.67 (t, 1H, $J = 8$ Hz, H-6), 7.75 (s, 1H, H-2), 8.18 (d, 1H, $J = 8$ Hz, H-8), 10.22 (s, 1H, D₂O exch, NHCO), 10.53 (t, $J = 5$ Hz, 1H, D₂O exch., NHCH₂); ¹³C-NMR (CDCl₃, 50 Hz) δ (ppm) 25.46 (COCH₃), 40.46 (NHCH₂CH₂N), 45.08 (N(CH₃)₂), 56.90 (NHCH₂CH₂N), 94.01 (C-2), 102.09 (C-9a), 117.59 (C-5), 121.42 (C-8a), 124.96 (C-7), 125.59 (C-8), 134.72 (C-6), 139.49 (C-3), 153.92 (C-1), 154.14 (C-10a), 154.23 (C-4a), 169.54 (COCH₃), 177.92 (C-9). Anal. (C₁₉H₂₀N₄O₅) C, H, N.

***N*-[1-[2-(Diethylamino)ethyl]amino-4-nitro-9-oxo-9*H*-xanthen-3-yl]acetamide**

(11b). This compound was prepared by a procedure analogous to that of **11a**. Yield: 92%; mp 177-179 °C (EtOAc - *n*-hexane); ¹H-NMR (CDCl₃, 400 MHz) δ (ppm) 1.07

(t, 6H, $J=7$ Hz, $\text{N}(\text{CH}_2\text{CH}_3)_2$), 2.24 (s, 3H, COCH_3), 2.61 (q, 4H, $J=7$ Hz, $\text{N}(\text{CH}_2\text{CH}_3)_2$), 2.76 (t, 2H, $J=7$ Hz, $\text{NHCH}_2\text{CH}_2\text{N}$), 3.22 (q, 2H, $J=7$ Hz, 5 Hz, $\text{NHCH}_2\text{CH}_2\text{N}$), 7.32 (t, 1H, $J=8$ Hz, H-7), 7.38 (d, 1H, $J=8$ Hz, H-5), 7.63 (t, 1H, $J=8$ Hz, H-6), 7.68 (s, 1H, H-2), 8.12 (d, 1H, $J=8$ Hz, H-8), 10.20 (s, 1H, D_2O exch., NHCO), 10.47 (t, $J=5$ Hz, 1H, D_2O exch., NHCH_2); ^{13}C -NMR (CDCl_3 , 50 Hz) δ (ppm) 11.81 ($\text{N}(\text{CH}_2\text{CH}_3)_2$), 25.90 (COCH_3), 41.29 ($\text{NHCH}_2\text{CH}_2\text{N}$), 46.88 ($\text{N}(\text{CH}_2\text{CH}_3)_2$), 50.59 ($\text{NHCH}_2\text{CH}_2\text{N}$), 93.97 (C-2), 102.01 (C-9a), 117.22 (C-4), 117.65 (C-5), 121.58 (C-8a), 124.87 (C-7), 125.71 (C-8), 134.55 (C-6), 139.69 (C-3), 154.13 (C-1), 154.18 (C-10a), 154.29 (C-4a), 169.34 (COCH_3), 177.53 (C-9). Anal. ($\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_5$) C, H, N.

Data for 5-[2-(dimethylamino)ethyl]amino-2-methyl-1,6-dihydroxantheno[3,4-*d*]imidazol-6-one (13a). Yield 82%; mp (difumarate) 217-219 $^{\circ}\text{C}$ (EtOH); ^1H -NMR (CDCl_3 , 400 MHz) δ (ppm) 2.30 (s, 6H, $\text{N}(\text{CH}_3)_2$), 2.57 (s, 3H, 2- CH_3), 2.65 (t, 2H, $J=7$ Hz, $\text{NHCH}_2\text{CH}_2\text{N}$), 3.21 (q, 2H, $J=7$ Hz, 5 Hz, $\text{NHCH}_2\text{CH}_2\text{N}$), 6.20 (s, 1H, H-4), 7.27 (t, 1H, $J=8$ Hz, H-8), 7.34 (d, 1H, $J=8$ Hz, H-10), 7.53 (t, 1H, $J=8$ Hz, H-9), 8.22 (d, 1H, $J=8$ Hz, H-7), 9.29 (t, $J=5$ Hz, 1H, D_2O exch., NHCH_2); ^{13}C -NMR (CDCl_3 , 50 Hz) δ (ppm) 14.96 (2- CH_3), 41.31 ($\text{NHCH}_2\text{CH}_2\text{N}$), 45.45 ($\text{N}(\text{CH}_3)_2$), 57.78 ($\text{NHCH}_2\text{CH}_2\text{N}$), 85.07 (C-4), 103.80 (C-5a), 117.26 (C-10), 121.52 (C-11b), 122.38 (C-6a), 123.79 (C-8), 126.01 (C-7), 133.80 (C-9), 143.03 (C-3a), 148.03, 148.36 (C-5, C-11a) 151.00 (C-2), 154.78 (C-10a), 179.26 (C-6). Anal. ($\text{C}_{19}\text{H}_{20}\text{N}_4\text{O}_2 \cdot 2\text{C}_4\text{H}_4\text{O}_4 \cdot 2\text{H}_2\text{O}$) C, H, N.

Data for 5-[2-(diethylamino)ethyl]amino-2-methyl-1,6-dihydroxantheno[3,4-*d*]imidazol-6-one (13b). Yield: 81%; mp (difumarate) 162-164 $^{\circ}\text{C}$ (EtOH); ^1H -NMR

(CDCl₃, 400 MHz) δ (ppm) 1.15 (t, 6H, $J=7$ Hz, N(CH₂CH₃)₂), 2.63 (s, 3H, 2-CH₃), 2.81 (q, 4H, $J=7$ Hz, N(CH₂CH₃)₂), 2.91 (t, 2H, $J=7$ Hz, NHCH₂CH₂N), 3.40 (q, 2H, $J=7$ Hz, 5 Hz, NHCH₂CH₂N), 6.40 (s, 1H, H-4), 7.33 (td, 1H, $J=8$ Hz, 2 Hz, H-8), 7.42 (d, 1H, $J=8$ Hz, H-10), 7.63 (td, 1H, $J=8$ Hz, 2 Hz, H-9), 8.27 (dd, 1H, $J=8$ Hz, 2 Hz, H-7), 9.36 (t, $J=5$ Hz, 1H, D₂O exch., NHCH₂); ¹³C-NMR (CDCl₃, 50 Hz) δ (ppm) 10.10 (N(CH₂CH₃)₂), 14.89 (2-CH₃), 40.25 (NHCH₂CH₂N), 46.87 (N(CH₂CH₃)₂), 50.64 (NHCH₂CH₂N), 85.18 (C-4), 103.92 (C-5a), 117.44 (C-10), 122.28 (C-6a), 123.71 (C-8), 125.90 (C-7), 133.80 (C-9), 142.52 (C-3a), 147.47, 147.48 (C-5, C-11a), 150.40 (C-2), 154.86 (C-10a), 179.37 (C-6). Anal. (C₂₁H₂₄N₄O₂ · 2C₄H₄O₄ · 1.25H₂O) C, H, N.

***N,N*-Dimethyl-*N'*-(3-amino-4-nitro-9-oxo-9*H*-xanthen-1-yl)ethane-1,2-diamine**

(14a). A solution of sodium hydroxide 40% (1 mL) was added to a stirred suspension of **11a** (340 mg, 1 mmol) in ethanol (10 mL) and the resulting mixture was heated at 60 °C for 1h. The solvent was then vacuum-evaporated and the resulting aqueous layer was extracted with CH₂Cl₂ (3x40 mL). The organic extracts were washed with brine, dried (Na₂SO₄) and concentrated to dryness. The residue was purified by column chromatography on silica gel, using a mixture of CH₂Cl₂:MeOH 100:3 as the eluent, to provide compound **14a** (318 mg, 93%); mp 193-195 °C (EtOAc - *n*-hexane); ¹H-NMR (CDCl₃, 400 MHz) δ (ppm) 2.33 (s, 6H, N(CH₃)₂), 2.64 (t, 2H, $J=7$ Hz, NHCH₂CH₂N), 3.28 (q, 2H, $J=7$ Hz, 5 Hz, NHCH₂CH₂N), 5.24 (s, 1H, H-2), 6.24 (s, 2H, D₂O exch., NH₂), 7.37 (t, 1H, $J=8$ Hz, H-7), 7.51 (d, 1H, $J=8$ Hz, H-5), 7.67 (t, 1H, $J=8$ Hz, H-6), 8.20 (d, 1H, $J=8$ Hz, H-8), 10.24 (t, $J=5$ Hz, 1H, D₂O exch., NHCH₂); ¹³C-NMR (CDCl₃, 50 Hz) δ (ppm) 40.94 (NHCH₂CH₂N), 45.52 (N(CH₃)₂), 57.38 (NHCH₂CH₂N), 87.63 (C-2), 100.81 (C-9a), 116.06 (C-4), 117.82

(C-5), 121.88 (C-8a), 124.83 (C-7), 125.60 (C-8), 134.19 (C-6), 149.79 (C-3), 153.18 (C-1), 154.26 (C-10a), 156.00 (C-4a), 177.71 (C-9). Anal. (C₁₇H₁₈N₄O₄) C, H, N.

***N,N*-Diethyl-*N'*-(3-amino-4-nitro-9-oxo-9*H*-xanthen-1-yl)ethane-1,2-diamine**

(14b). This compound was prepared by a procedure analogous to that of **14a**. Yield: 92%; mp 128-130 °C (EtOAc - *n*-hexane); ¹H-NMR (CDCl₃, 400 MHz) δ (ppm) 1.01 (t, 6H, *J* = 7 Hz, N(CH₂CH₃)₂), 2.55 (q, 4H, *J* = 7 Hz, N(CH₂CH₃)₂), 2.67 (t, 2H, *J* = 7 Hz, NHCH₂CH₂N), 3.12 (q, 2H, *J* = 7 Hz, 5 Hz, NHCH₂CH₂N), 5.48 (s, 1H, H-2), 6.48 (s, 2H, D₂O exch., NH₂), 7.26 (t, 1H, *J* = 8 Hz, H-7), 7.37 (d, 1H, *J* = 8 Hz, H-5), 7.56 (t, 1H, *J* = 8 Hz, H-6), 8.05 (d, 1H, *J* = 8 Hz, H-8), 10.01 (t, *J* = 5 Hz, 1H, D₂O exch., NHCH₂); ¹³C-NMR (CDCl₃, 50 Hz) δ (ppm) 11.58 (N(CH₂CH₃)₂), 41.02 (NHCH₂CH₂N), 46.88 (N(CH₂CH₃)₂), 50.78 (NHCH₂CH₂N), 87.69 (C-2), 100.55 (C-9a), 115.72 (C-4), 117.60 (C-5), 121.75 (C-8a), 124.67 (C-7), 125.40 (C-8), 133.96 (C-6), 150.03 (C-3), 152.85 (C-1), 154.02 (C-10a), 155.84 (C-4a), 177.17 (C-9). Anal. (C₁₉H₂₂N₄O₄) C, H, N.

Data for 5-[2-(dimethylamino)ethyl]amino-1,6-dihydroxantheno[3,4-*d*]imidazol-6-one (16a). Yield 80%; mp (dihydrochloride) 256-258 °C (EtOH); ¹H-NMR (CDCl₃, 400 MHz) δ (ppm) 2.37 (s, 6H, N(CH₃)₂), 2.73 (t, 2H, *J* = 7 Hz, NHCH₂CH₂N), 3.30 (q, 2H, *J* = 7 Hz, 5 Hz, NHCH₂CH₂N), 6.32 (s, 1H, H-4), 7.33 (t, 1H, *J* = 8 Hz, H-8), 7.48 (d, 1H, *J* = 8 Hz, H-10), 7.61 (t, 1H, *J* = 8 Hz, H-9), 7.94 (s, 1H, H-2), 8.26 (d, 1H, *J* = 8 Hz, H-7), 9.37 (t, *J* = 5 Hz, 1H, D₂O exch., NHCH₂); ¹³C-NMR (CDCl₃, 50 Hz) δ (ppm) 41.13 (NHCH₂CH₂N), 45.35 (N(CH₃)₂), 57.25 (NHCH₂CH₂N), 84.84 (C-4), 104.35 (C-5a), 117.45 (C-10), 121.74 (C-11b), 122.37 (C-6a), 123.96 (C-8),

125.98 (C-7), 133.95 (C-9), 140.17 (C-2), 141.18 (C-3a), 148.07 (C-5), 149.68 (C-11a), 154.82 (C-10a), 179.50 (C-6). Anal. (C₁₈H₁₈N₄O₂ · 2HCl · 2H₂O) C, H, N.

Data for 5-[2-(diethylamino)ethyl]amino-1,6-dihydroxantheno[3,4-*d*]imidazol-6-one (16b). Yield: 81%; mp (dihydrochloride) 247-249 °C (EtOH); ¹H-NMR (CDCl₃, 400 MHz) δ (ppm) 1.20 (t, 6H, *J*= 7 Hz, N(CH₂CH₃)₂), 2.85 (q, 4H, *J*= 7 Hz, N(CH₂CH₃)₂), 2.98 (t, 2H, *J*= 7 Hz, NHCH₂CH₂N), 3.43 (q, 2H, *J*= 7 Hz, 5 Hz, NHCH₂CH₂N), 6.54 (s, 1H, H-4), 7.35 (td, 1H, *J*= 8 Hz, 2 Hz, H-8), 7.56 (d, 1H, *J*= 8 Hz, H-10), 7.65 (td, 1H, *J*= 8 Hz, 2 Hz, H-9), 7.97 (s, 1H, H-2), 8.26 (dd, 1H, *J*= 8 Hz, 2 Hz, H-7), 9.38 (t, *J*=5 Hz, 1H, D₂O exch., NHCH₂); ¹³C-NMR (CDCl₃, 50 Hz) δ (ppm) 10.87 (N(CH₂CH₃)₂), 41.12 (NHCH₂CH₂N), 47.04 (N(CH₂CH₃)₂), 51.09 (NHCH₂CH₂N), 85.14 (C-4), 104.38 (C-5a), 117.41 (C-10), 122.08 (C-11b), 122.41 (C-6a), 123.96 (C-8), 126.04 (C-7), 133.93 (C-9), 140.29 (C-2), 141.09 (C-3a), 148.29 (C-5), 149.75 (C-11a), 154.76 (C-10a), 179.34 (C-6). Anal. (C₂₀H₂₂N₄O₂ · 2HCl · 2H₂O) C, H, N.

***N*-[2-(2-Hydroxyethyl)-5-nitro-2*H*-chromeno[4,3,2-*c,d*]indazol-4-yl]acetamide (18).**

Method A

A solution of **10** (680 mg, 2.04 mmol) and 2-hydroxyethylhydrazine (1.14 ml, 20.45 mmol) in dry DMSO (4 mL) was stirred at room temperature for 1h. The reaction mixture was then poured into ice/water and extracted with CH₂Cl₂ (3x40 mL). The combined organic extracts were washed with brine, dried (Na₂SO₄) and evaporated to dryness. The residue was purified by column chromatography on silica gel, eluting

with EtOAc:cyclohexane 1:1, to provide compounds **17** (250 mg, 32%) and **18** (14 mg, 2%).

Method B

To a suspension of **10** (860 mg, 2.59 mmol) in dry THF (40 mL), were added 4-dimethylaminopyridine (348 mg, 2.84 mmol) and a solution of 2-hydroxyethylhydrazine (0.193 mL, 2.84 mmol) in absolute ethanol and the mixture was stirred at room temperature for 20h. The solvents were vacuum-evaporated, the residue was acidified with a 9% HCl solution and the precipitate was filtered, washed with water, and air-dried, to provide the carbinol **18** (870 mg, 95%).

Data for N-[2-(2-hydroxyethyl)-5-[1-(2-hydroxyethyl)hydrazino]-2H-chromeno[4,3,2-c,d]indazol-4-yl]acetamide (17): mp >270 °C (MeOH); ¹H-NMR (DMSO-*d*₆, 400 MHz) δ (ppm) 2.25 (s, 3H, COCH₃), 3.86 (q, 2H, *J*= 5 Hz, 5 Hz, NNCH₂CH₂OH), 3.98 (q, 2H, *J*= 5 Hz, 5Hz, NH₂NCH₂CH₂OH), 4.48 (t, 2H, *J*= 5 Hz, NNCH₂CH₂OH), 4.71 (t, 2H, *J*= 5 Hz, NH₂NCH₂CH₂OH), 5.01 (t, 1H, *J*= 5 Hz, D₂O exch., NNCH₂CH₂OH), 5.10 (t, 1H, *J*= 5 Hz, D₂O exch., NH₂NCH₂CH₂OH), 7.00-7.07 (m, 2H, H-7, H-9), 7.31 (td, 1H, *J*= 8 Hz, 2 Hz, H-8), 8.33 (s, 1H, H-3), 8.87 (dd, 1H, *J*= 8 Hz, 2 Hz, H-10), 10.48 (s, 1H, D₂O exch., NH), 11.06 (s, 2H, D₂O exch., NH₂); ¹³C-NMR (DMSO-*d*₆, 50 Hz) δ (ppm) 25.00 (COCH₃), 51.68 (NH₂NCH₂CH₂OH), 52.27 (NNCH₂CH₂OH), 58.19 (NH₂NCH₂CH₂OH), 60.23 (NNCH₂CH₂OH), 97.73 (C-3), 103.74 (C-2b), 115.66 (C-5), 116.74 (C-10a), 117.05 (C-7), 119.60 (C-9), 127.00 (C-5a), 129.68 (C-10), 130.17 (C-8), 134.63 (C-4), 141.84 (C-2a), 144.14 (C-10b), 156.24 (C-6a), 169.54 (NHCO). Anal. (C₁₉H₂₁N₅O₄) C, H, N.

Data for N-[2-(2-hydroxyethyl)-5-nitro-2H-chromeno[4,3,2-c,d]indazol-4-yl]acetamide (18): mp 256-258 °C (DMF-H₂O); ¹H-NMR (DMSO-*d*₆, 400 MHz) δ

(ppm) 2.04 (s, 3H, COCH₃), 3.83 (q, 2H, *J*= 5 Hz, 5 Hz, NCH₂CH₂OH), 4.41 (t, 2H, *J*= 5 Hz, NCH₂CH₂OH), 4.95 (t, 1H, *J*= 5 Hz, D₂O exch., NCH₂CH₂OH), 7.18 (s, 1H, H-3), 7.40 (td, 1H, *J*= 8 Hz, 2 Hz, H-8), 7.57-7.48 (m, 2H, H-7, H-9), 7.91 (dd, 1H, *J*= 8 Hz, 2 Hz, H-10), 10.29 (s, 1H, D₂O exch., NH); ¹³C-NMR (DMSO-d₆, 50 Hz) δ (ppm) 23.64 (COCH₃), 52.83 (NNCH₂CH₂OH), 60.60 (NNCH₂CH₂OH), 100.37 (C-3), 112.58 (C-2b), 117.32 (C-10a), 119.26 (C-7), 123.35 (C-10), 123.60 (C-5), 126.50 (C-9), 131.54 (C-8), 133.60 (C-4), 138.85 (C-10b), 140.52 (C-2a), 144.56 (C-5a), 153.68 (C-6a), 169.26 (NHCO). Anal. (C₁₇H₁₄N₄O₅) C, H, N.

2-(4-Acetylamino-5-nitro-2*H*-chromeno[4,3,2-*c,d*]indazol-2-yl)ethyl

methanesulfonate (19). A solution of methanesulfonyl chloride (121 μL, 1.55 mmol) in CH₂Cl₂ (5 mL) was added dropwise at 0 °C to a suspension of **18** (500 mg, 1.412 mmol) in dry pyridine (6 mL) and the mixture was stirred at room temperature for 10 min. The reaction mixture was poured into ice/water, acidified with hydrochloric acid 9% and the resulting solid was filtered, washed with water, and air-dried, to give compound **19** (550 mg, 90%); mp 207-209 °C (EtOH); ¹H-NMR (DMSO-*d*₆, 400 MHz) δ (ppm) 2.01 (s, 3H, COCH₃), 3.06 (s, 3H, SO₂CH₃), 4.60 (t, 2H, *J*= 5 Hz, NNCH₂CH₂O), 4.71 (t, 2H, *J*= 5 Hz, NNCH₂CH₂O), 7.20 (s, 1H, H-3), 7.36 (td, 1H, *J*= 8 Hz, 2 Hz, H-9), 7.44-7.54 (m, 2H, H-7, H-8), 7.88 (dd, 1H, *J*= 8 Hz, 2 Hz, H-10), 10.27 (s, 1H, D₂O exch., NH); ¹³C-NMR (DMSO-*d*₆, 50 Hz) δ (ppm) 23.56 (COCH₃), 37.12 (SO₂CH₃), 48.89 (NNCH₂CH₂O), 68.66 (NNCH₂CH₂O), 99.87 (C-3), 112.56 (C-2b), 116.97 (C-10a), 119.19 (C-7), 123.38 (C-10), 123.82 (C-5), 126.42 (C-9), 131.68 (C-8), 133.94 (C-4), 139.40 (C-10b), 140.37 (C-2a), 144.43 (C-5a), 153.70 (C-6a), 169.19 (NHCO). Anal. (C₁₈H₁₆N₄O₇S) C, H, N.

2-(9-Methyl-6,8-dihydrochromeno[4,3,2-*c,d*]imidazo[4,5-*f*]indazol-6-yl)ethyl methanesulfonate (21). This compound was prepared by a procedure analogous to that of **13a**. Yield 84%; mp >270 °C (EtOAc - *n*-hexane); ¹H-NMR (CDCl₃, 400 MHz) δ (ppm) 2.72 (s, 3H, 9-CH₃), 2.80 (s, 3H, SO₂CH₃), 4.44 (t, 2H, *J*= 5 Hz, NCH₂CH₂O), 4.62 (t, 2H, *J*= 5 Hz, NCH₂CH₂O), 6.79 (s, 1H, H-7), 7.07-7.15 (m, 2H, H-1, H-2), 7.21 (t, 1H, *J*= 8 Hz, H-3), 7.67 (d, 1H, *J*= 8 Hz, H-4); ¹³C-NMR (CDCl₃, 50 Hz) δ (ppm) 14.19 (9-CH₃), 37.26 (SO₂CH₃), 48.34 (NNCH₂CH₂O), 68.31 (NNCH₂CH₂O), 82.89 (C-7), 113.52 (C-6b), 117.62 (C-10a), 117.84 (C-4a), 118.22 (C-1), 122.98 (C-4), 124.74 (C-3), 130.24 (C-2), 135.54 (C-7a or C-10b), 138.52 (C-6a), 138.63 (C-6c), 139.60 (C-7a or C-10b), 152.44 (C-9), 154.07 (C-11a). Anal. (C₁₈H₁₆N₄O₄S) C, H, N.

Data for *N,N*-dimethyl-*N*-[2-(9-methyl-6,8-dihydrochromeno[4,3,2-*c,d*]imidazo[4,5-*f*]indazol-6-yl)ethyl]amine (22a). Yield 92%; mp (dihydrochloride) >270 °C (EtOH); ¹H-NMR (CDCl₃, 400 MHz) δ (ppm) 2.49 (s, 6H, N(CH₃)₂), 2.56 (s, 3H, 9-CH₃), 3.14 (t, 2H, *J*= 7 Hz, NNCH₂CH₂N), 4.50 (t, 2H, *J*= 7 Hz, NNCH₂CH₂N), 6.65 (s, 1H, H-7), 7.21-7.28 (m, 2H, H-1, H-2), 7.37 (t, 1H, *J*= 8 Hz, H-3), 7.86 (d, 1H, *J*= 8 Hz, H-4); ¹³C-NMR (CDCl₃, 50 Hz) δ (ppm) 15.20 (9-CH₃), 45.23 (N(CH₃)₂), 46.92 (NNCH₂CH₂N), 58.19 (NNCH₂CH₂N), 83.13 (C-7), 113.14 (C-6b), 118.19 (C-1), 118.77 (C-4a), 120.33 (C-10a), 122.65 (C-4), 124.12 (C-3), 129.69 (C-2), 135.01 (C-7a or C-10b), 137.43 (C-6a), 137.73 (C-6c), 143.96 (C-7a or C-10b), 152.66 (C-9), 154.59 (C-11a). Anal. (C₁₉H₁₉N₅O · 2HCl · 2H₂O) C, H, N.

Data for *N,N*-diethyl-*N*-[2-(9-methyl-6,8-dihydrochromeno[4,3,2-*c,d*]imidazo[4,5-*f*]indazol-6-yl)ethyl]amine (22b). Yield: 90%; mp (dihydrochloride) >270 °C

(EtOH); $^1\text{H-NMR}$ (CDCl_3 , 400 MHz) δ (ppm) 1.06 (t, 6H, $J=7$ Hz, $\text{N}(\text{CH}_2\text{CH}_3)_2$), 2.60 (s, 3H, 9- CH_3), 2.75 (q, 4H, $J=7$ Hz, $\text{N}(\text{CH}_2\text{CH}_3)_2$), 3.12 (t, 2H, $J=7$ Hz, $\text{NNCH}_2\text{CH}_2\text{N}$), 4.44 (t, 2H, $J=7$ Hz, $\text{NNCH}_2\text{CH}_2\text{N}$), 6.73 (s, 1H, H-7), 7.14 (t, 1H, $J=8$ Hz, H-3), 7.20-7.30 (m, 2H, H-1, H-2), 7.82 (d, 1H, $J=8$ Hz, H-4); $^{13}\text{C-NMR}$ (CDCl_3 , 50 Hz) δ (ppm) 10.71 ($\text{N}(\text{CH}_2\text{CH}_3)_2$), 15.34 (9- CH_3), 46.98 ($\text{N}(\text{CH}_2\text{CH}_3)_2$), 47.05 ($\text{NNCH}_2\text{CH}_2\text{N}$), 51.62 ($\text{NNCH}_2\text{CH}_2\text{N}$), 81.97 (C-7), 113.35 (C-6b), 118.48 (C-1), 118.64 (C-4a), 122.32 (C-10a), 122.91 (C-4), 124.32 (C-3), 129.91 (C-2), 136.80 (C-7a or C-10b), 138.01 (C-6a), 138.40 (C-6c), 142.06 (C-7a or C-10b), 152.35 (C-9), 154.65 (C-11a). Anal. ($\text{C}_{21}\text{H}_{23}\text{N}_5\text{O} \cdot 2\text{HCl} \cdot 3.5\text{H}_2\text{O}$) C, H, N.

2-[4-Amino-5-nitro-2H-chromeno[4,3,2-c,d]indazol-2-yl]-1-ethanol (23). HCl 9% (1 mL) was added to a stirred solution of **18** (400 mg, 1.13 mmol) in dioxane (15 mL) and the resulting mixture was heated at reflux for 3h. The solvent was vacuum-evaporated and the resulting aqueous layer was extracted with CH_2Cl_2 (3x70 mL). The organic extracts were dried (Na_2SO_4) and concentrated to dryness. The resulting solid was recrystallized from ethanol to yield compound **23** (330 mg, 94%); mp >270 $^\circ\text{C}$ (EtOH); $^1\text{H-NMR}$ ($\text{DMSO-}d_6$, 400 MHz) δ (ppm) 3.80 (t, 2H, $J=5$ Hz, $\text{NNCH}_2\text{CH}_2\text{OH}$), 4.20 (t, 2H, $J=5$ Hz, $\text{NNCH}_2\text{CH}_2\text{OH}$), 6.28 (s, 1H, H-3), 7.00 (s, 2H, D_2O exch., NH_2), 7.42 (t, 1H, $J=8$ Hz, H-9), 7.54-7.59 (m, 2H, H-7, H-8), 7.90 (d, 1H, $J=8$ Hz, H-10); $^{13}\text{C-NMR}$ ($\text{DMSO-}d_6$, 50 Hz) δ (ppm) 52.10 ($\text{NNCH}_2\text{CH}_2\text{O}$), 60.20 ($\text{NNCH}_2\text{CH}_2\text{O}$), 85.69 (C-3), 108.76 (C-2b), 117.33 (C-10a), 117.80 (C-5), 119.38 (C-7), 123.19 (C-10), 126.46 (C-9), 131.32 (C-8), 139.02 (C-10b), 142.68 (C-2a), 148.27 (C-4, C-5a), 153.64 (C-6a). Anal. ($\text{C}_{15}\text{H}_{12}\text{N}_4\text{O}_4$) C, H, N.

2-(4-Amino-5-nitro-2H-chromeno[4,3,2-c,d]indazol-2-yl)ethyl methanesulfonate (24). This compound was prepared according to the procedure described for the preparation of **19**, starting from **23**. Yield: 96%; mp 197-199 °C (EtOH); ¹H-NMR (DMSO-*d*₆, 400 MHz) δ (ppm) 3.06 (s, 3H, SO₂CH₃), 4.48 (t, 2H, *J* = 5 Hz, NNCH₂CH₂O), 4.57 (t, 2H, *J* = 5 Hz, NNCH₂CH₂O), 6.25 (s, 1H, H-3), 7.06 (s, 1H, D₂O exch., NH₂), 7.38 (td, 1H, *J* = 8 Hz, 2 Hz, H-9), 7.50-7.55 (m, 2H, H-7, H-8), 7.86 (dd, 1H, *J* = 8 Hz, 2 Hz, H-10); ¹³C-NMR (DMSO-*d*₆, 50 Hz) δ (ppm) 37.16 (SO₂CH₃), 48.44 (NNCH₂CH₂O), 68.74 (NNCH₂CH₂O), 85.45 (C-3), 108.29 (C-2b), 117.13 (C-10a), 118.11 (C-5), 119.47 (C-7), 123.36 (C-10), 126.54 (C-9), 131.63 (C-8), 139.70 (C-10b), 142.78 (C-2a), 148.69 (C-4, C-5a), 153.80 (C-6a). Anal. (C₁₆H₁₄N₄O₆S) C, H, N.

2-(6,8-Dihydrochromeno[4,3,2-c,d]imidazo[4,5-f]indazol-6-yl)ethyl methanesulfonate (26). This compound was prepared according to the procedure described for the preparation of **16**, starting from **24**. Compound **26** (yield 83%) resulted as a mixture of both tautomers. ¹H-NMR (CDCl₃, 400 MHz, chemical shifts in parentheses represent the minor tautomer) δ (ppm) 2.64 (2.66) (s, 3H, SO₂CH₃), 4.48 (m, 2H, NCH₂CH₂O), 4.60 (m, 2H, NCH₂CH₂O), 6.67 (6.77) (s, 1H, H-7), 7.12 (m, 1H, H-3), 7.21-7.29 (m, 2H, H-1, H-2), 7.72-7.78 (m, 1H, H-4), 8.04 (s, 1H, H-9). Anal. (C₁₇H₁₄N₄O₄S) C, H, N.

***N,N*-Dimethyl-*N*-[2-(6,8-dihydrochromeno[4,3,2-c,d]imidazo[4,5-f]indazol-6-yl)ethyl]amine (27a)**. This compound was prepared according to the procedure described for the preparation of **22**, starting from **26**. Yield: 91%; ¹H-NMR (CDCl₃, 400 MHz, chemical shifts in parentheses represent the minor tautomer) δ (ppm) 2.36

(2.42) (s, 6H, N(CH₃)₂), 2.92 (2.98) (t, 2H, *J* = 7 Hz, NNCH₂CH₂N), 4.37 (m, 2H, NNCH₂CH₂N), 6.54 (6.67) (s, 1H, H-7), 7.12 (m, 1H, H-3), 7.16-7.27 (m, 2H, H-1, H-2), 7.74-7.82 (m, 2H, H-4, H-9); ¹³C-NMR (CDCl₃, 50 Hz) δ (ppm) 45.16 (45.32) (N(CH₃)₂), 46.88 (47.20) (NNCH₂CH₂N), 58.11 (NNCH₂CH₂N), 82.76 (C-7), 113.14 (113.58) (C-6b), 118.15 (118.29) (C-1), 118.62 (118.71) (C-4a), 122.67, 122.83, 123.07 (C-4, C-10a), 124.14 (124.28) (C-3), 129.70 (129.75) (C-2), 137.06, 137.47, 137.80, 138.02, 138.17 (C-6a, C-6c, C-7a, C-10b), 142.09 (C-9), 154.49 (154.53) (C-11a). Anal. (C₁₈H₁₇N₅O · 2C₄H₄O₄ · 2H₂O) C, H, N.

***N,N*-Diethyl-*N*-[2-(6,8-dihydrochromeno[4,3,2-*c,d*]imidazo[4,5-*f*]indazol-6-**

yl)ethyl]amine (27b). This compound was prepared according to the procedure described for the preparation of **22**, starting from **26**. Yield: 83%; mp (difumarate) 177-179 °C (EtOH); ¹H-NMR (CDCl₃, 400 MHz) δ (ppm) 1.02 (t, 6H, *J* = 7 Hz, N(CH₂CH₃)₂), 2.08 (q, 4H, *J* = 7 Hz, N(CH₂CH₃)₂), 3.45 (t, 2H, *J* = 7 Hz, NNCH₂CH₂N), 4.37 (t, 2H, *J* = 7 Hz, NNCH₂CH₂N), 6.77 (s, 1H, H-7), 7.09 (t, 1H, *J* = 8 Hz, H-3), 7.16-7.24 (m, 2H, H-1, H-2), 7.78 (d, 1H, *J* = 8 Hz, H-4), 8.00 (s, 1H, H-9); ¹³C-NMR (CDCl₃, 50 Hz) δ (ppm) 10.57 (N(CH₂CH₃)₂), 46.70 (N(CH₂CH₃)₂), 46.89 (NNCH₂CH₂N), 51.47 (NNCH₂CH₂N), 82.41 (C-7), 113.47 (C-6b), 118.19 (C-1), 118.42 (C-4a), 121.98 (C-10a), 122.87 (C-4), 124.33 (C-3), 129.79 (C-2), 137.54 (C-7a or C-10b), 138.18 (C-6c), 138.29 (C-6a), 140.66 (C-7a or C-10b), 142.16 (C-9), 154.36 (C-11a). Anal. (C₂₀H₂₁N₅O · 2C₄H₄O₄) C, H, N.

Biological Evaluation

Materials. Eagle's minimal essential medium (EMEM), Foetal bovine serum (FBS), sodium pyruvate, sodium bicarbonate, L-glutamine, nonessential amino acids,

penicillin, streptomycin, amphoterecin B and gentamycin were all obtained from Biochrom KG (Berlin, Germany). Insulin was obtained from Sigma Chemicals (Steinheim, Germany). All other chemicals used were of the best commercially available grade.

Cell culture conditions. MDA-MB-231 (HTB 26: human breast adenocarcinoma, ER-negative, high invasive potential) was obtained from the American Type Culture Collection (ATCC) and cultured as monolayers at 37°C in a humidified atmosphere of 5% (v/v) CO₂ and 95% air. The cells were seeded in 75-cm² plastic tissue culture flasks and cultured in EMEM supplemented with 10% FBS, 2 mM L-glutamine, 1.0 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 0.01 mg/mL of insulin and a cocktail of antimicrobial agents (100 IU/mL penicillin, 100 µg/mL streptomycin, 10 µg/mL gentamicin sulphate and 2.5 µg/mL amphoterecin B).¹² According to pilot experiments in respect to growth rate and doubling time, the medium was changed every three days. The cells were harvested after treatment with 0.25% (w/v) trypsin in PBS, containing 0.1% (w/v) Na₂EDTA.

Cell proliferation. In order to evaluate the effects of the new aminoderivatives on cell proliferation, the cells were seeded in the presence of serum into 96-well plates at a density of 1 x 10⁴ cells per well. Twenty four hours after plating, new medium supplemented with the aminoderivatives to be tested at the following concentrations was added. The tested compounds were diluted in DMSO and/or ethanol and stored at -20 °C. To achieve the target concentrations for the experiments, the stock reagents were diluted to appropriate final concentrations in the culture media. After 72 h incubation, the medium was replaced with WST-1 (water-soluble tetrazolium salt).

Subsequently the cells were incubated for 3 h and the quantification of the formazan dye in the microplate was measured with an ELISA plate reader at 450 nm (reference wavelength at 650 nm). IC_{50} represents the concentration that reduces by 50% the optical density of treated cells with respect to untreated controls.