

## Supporting information

### Optimization of anti-tumor agent WMC79 leading to clinical candidate HKA40 (RTA 502)

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#### Supporting information

- Synthetic protocol for compounds **3a**, **4**, **5a-d**, **6b-d** and **7a-h**.
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#### Synthetic protocols

**General.** All commercial solvents and reagents were used without further purification. Naphthalic anhydrides were purchased from Aldrich. Melting points were determined on an Electrothermal capillary melting point apparatus and are uncorrected. <sup>1</sup>H NMR spectra were recorded on a Varian VXR-S spectrometers operating at 400 MHz and 500 MHz. Preparative reverse phase HPLC were performed using Xterra column (5 mm C18 as stationary phase and acetonitrile and water buffered with 0.1% of TFA as mobile phase). Elemental analyses were performed either on the free bases or as their TFA salts by Atlantic Microlab, Inc., Norcross, GA and Desert Analytics, Tuscon, AZ.

#### 6-Chloro-2-(4-fluorophenylamino)-3-nitro-benzoic acid (**4**)

A mixture of 2,6-dichloro-3-nitrobenzoic acid (18.88g, 0.08 mol), 4-fluoroaniline (26.8 g, 0.18 mol) and ethanol (50 mL) was heated at reflux for 30 hours. The solvent was evaporated and the residue was triturated with benzene (100 mL) and 2N NaOH solution (150 mL) ; the mixture was stirred thoroughly for 1 hour. Undissolved material was separated by filtration. The aqueous layer was separated and traces of benzene were removed by evaporation. The solution was then made acidic by addition of concentrated hydrochloric acid. The yellow precipitate was collected by filtration and washed with water (100 mL). After drying, the

crude material was crystallized from toluene to give 15.36 g (62%) of **4**: mp 216-220°C. Anal. (C<sub>13</sub>H<sub>7</sub>N<sub>2</sub>O<sub>4</sub>ClF) C, H, N.

### 1-Chloro-7-fluoro-4-nitro-10*H*-acridin-9-one (**3a**)

A mixture of **4** (12.39 g, 0.04 mol), chloroform (100 mL), and POCl<sub>3</sub> (60 mL, 0.64 mol) was heated at reflux for 8 h. All the volatiles were removed under reduced pressure and the residue was diluted with 200 mL of a mixture of 1,4-dioxane - water (8:1). The resulting mixture was acidified with concentrated hydrochloric acid and refluxed with stirring for 2 h. Water was added (200 mL), the precipitate was collected by filtration and crystallized from *N,N*-dimethylformamide - water to give 10.2 g (87%) of **3a** as orange needles: mp 287-291°C. Anal. (C<sub>13</sub>H<sub>6</sub>N<sub>2</sub>O<sub>3</sub>ClF) C, H, N.

### 1-{3-[4-(3-Amino-propyl)-piperazin-1-yl]-propylamino}-7-fluoro-4-nitro-10*H*-acridin-9-one (**5a**)

A mixture of **3a** (2.93 g, 0.01 mol), *N,N*-dimethylformamide (50 mL), and 1,4-bis(3-aminopropyl)piperazine (10.0 g, 0.05 mol) was stirred at room temperature for 20 h. Water (250 mL) was added to the reaction mixture, stirred thoroughly and left overnight in a refrigerator. The fine precipitate thus formed was collected by centrifugation, transferred into 2% aqueous hydrochloric acid (300 mL), and stirred for 2 h. Un-dissolved material was separated by centrifugation. The solution was made alkaline and the product was extracted with chloroform (3 x 100 mL). The chloroform extract was dried and evaporated. Crude product was crystallized from toluene-hexane to give 2.78 g (61%) of **5a** as a yellow solid: mp 130-134°C. Anal. (C<sub>23</sub>H<sub>29</sub>N<sub>6</sub>O<sub>3</sub>F) C, H, N.

### 1-{3-[4-(3-Aminopropyl)-piperazin-1-yl]-propylamino}-7-methoxy-4-nitro-10*H*-acridin-9-one (**5b**)

To a solution of 1,4-bis(3-aminopropyl)piperazine (50.0 g, 0.25 mol) in dimethyl sulfoxide (600 mL) was added 1-chloro-7-methoxy-4-nitro-10*H*-acridin-9-one **3b**<sup>1</sup> (14 g, 0.046 mol) in portions over 1h at 60°C under Argon. The resulting slurry was stirred at that temperature for 16 h, then diluted with water (4L) and cooled to about 10°C. The precipitate was collected by centrifugation, transferred into 2% aqueous hydrochloric acid (1.5L), and stirred for 1 h. Undissolved material was separated by centrifugation. The filtrate was made alkaline and the product was extracted with a 200:1 mixture of chloroform and methanol (3 x 700 mL). Combined organic extracts was dried and evaporated. Crude product was crystallized twice from toluene-hexane to give 14 g (65%) of **5b** as a yellow powder. <sup>1</sup>H NMR (CDCl<sub>3</sub>) 12.70 (s, 1H), 12.04 (br t, 1H), 8.43 (d, 1H), 7.72 (d, 1H), 7.42 (d, 1H), 7.34 (dd, 1H), 6.42 (d, 1H), 3.93 (s, 3H), 3.60-3.45 (m, 2H), 2.75 (t, 2H), 2.64-2.42 (m, 10H), 2.41 (t, 2H), 2.04-1.87 (m, 2H), 1.74-1.55 (m, 2H), 1.44 (br s, 2H). Anal. (C<sub>24</sub>H<sub>32</sub>N<sub>6</sub>O<sub>4</sub>.3/2H<sub>2</sub>O) C, H, N.

### **1-{3-[4-(3-Aminopropyl)-piperazin-1-yl]-propylamino}-7-methyl-4-nitro-10H-acridin-9-one (5c)**

To a solution of 1,4-bis(3-aminopropyl)piperazine (25.0 g, 0.125 mol) in dimethyl sulfoxide (200 mL) was added 1-chloro-7-methyl-4-nitro-10H-acridin-9-one **3c**<sup>1</sup> (6 g, 0.021 mol) in portions over 1h at 60°C under Argon. The resulting orange slurry was stirred at that temperature for 18 h, all the volatiles were removed under reduced pressure and diluted with water (600 mL). The suspension thus obtained was extracted with a 200:1 mixture of chloroform to methanol (3 x 200 mL). Combined organic extracts was dried and evaporated to dryness to give a residue which was chromatographed on basic alumina using 1% to 10% methanol in chloroform gradient. The resulting yellow solid was crystallized from toluene-hexane to give 6 g (64%) of **5c**; <sup>1</sup>H NMR (CDCl<sub>3</sub>) 12.61 (s, 1H), 12.01 (t, 1H), 8.41 (d, 1H), 8.12 (br s, 1H), 7.52 (dd, 1H), 7.35 (d, 1H), 6.42 (d, 1H), 3.54-3.48 (m, 2H), 2.75 (t, 2H), 2.55-2.44 (m, 13H), 2.44-2.38 (m, 2H), 2.00-1.91 (m, 2H), 1.69-1.60 (m, 2H), 1.45-1.18 (br s, 2H). Anal. (C<sub>24</sub>H<sub>32</sub>N<sub>6</sub>O<sub>3</sub>.1/2H<sub>2</sub>O) C, H, N.

### **1-{3-[4-(3-Aminopropyl)-piperazin-1-yl]-propylamino}-8-methyl-4-nitro-10H-acridin-9-one (5d)**

To a solution of 1,4-bis(3-aminopropyl)piperazine (8.33 g, 41.58 mmol) in dimethyl sulfoxide (50 mL) was added 1-chloro-7-methyl-4-nitro-10H-acridin-9-one **3d**<sup>1</sup> (2 g, 6.93 mmol) in portions over 1h at 60°C under Argon. The resulting orange slurry was stirred at that temperature for 18 h, all the volatiles were then removed under reduced pressure and diluted with water (200 mL). The suspension thus obtained was extracted with a 200:1 mixture of chloroform to methanol (3 x 100 mL). Combined organic extracts was dried and evaporated to dryness to give a residue which was chromatographed on basic alumina using 1% to 10% methanol in chloroform gradient as an eluent. The resulting yellow solid was crystallized from toluene-hexane to give 1.88 g (60%) of **5d**. Anal. (C<sub>24</sub>H<sub>32</sub>N<sub>6</sub>O<sub>3</sub>.TFA.H<sub>2</sub>O) C, H, N.

### **5-{3-[4-(3-Amino-propyl)-piperazin-1-yl]-propylamino}-8-fluoro-2,10b-diaza-aceanthrylen-6-one (6a)**

A solution of **5a** (1.37 g, 0.003 mol) in 88% formic acid (30 mL) was hydrogenated over Raney Ni (about 0.8 g) under 1 atmosphere of H<sub>2</sub> overnight. The reaction mixture was filtered to remove the catalyst. Concentrated hydrochloric acid (3 mL) was added to the filtrate and the mixture was heated at reflux for 8 hours. The reaction mixture was condensed to about 10 mL and the product was precipitated as a salt by addition of acetone (50 mL). After drying the precipitate was dissolved in water (10 mL) and chromatographed on preparative HPLC reverse phase column with a gradient of 0.5% TFA in water:methanol (95:5 to 40:60). The major fraction was collected, made alkaline and the product was extracted with chloroform (3 x 100 mL) as a free base. After evaporation of solvent, the product was crystallized from benzene-hexane to give 0.741 g (59%) of **6a** as a yellow crystalline powder: mp 126-130°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.99 (t, 1H), 8.50 (s, 1H), 8.20 (m, 1H), 7.97 (d, 1H), 7.92 (m, 1H), 7.52 (m, 1H, C9-H), 6.81 (d, J = 9.0 Hz, 1H, C4-H), 3.50 (m, 2H), 2.75 (t, 2H), 2.52 (m, 10H), 2.42 (t, 2H), 1.96 (m, 2H), 1.65 (m, 2H). Anal. (C<sub>24</sub>H<sub>29</sub>N<sub>6</sub>OF) C, H, N.

**General procedure for the preparation of 6b-d** (For the reductive cyclization of ~10 mmol of **5b-d**):

A warm solution of SnCl<sub>2</sub> (100 mmol) in concentrated hydrochloric acid (30 mL) was added to a stirred solution of **5b-d** (10 mmol) in 88% formic acid (175 mL) at 60°C such that the precipitation did not stop the stirring. The resulting yellow slurry was refluxed for 20h. After complete disappearance of the starting material (TLC), the reaction mixture was condensed to about 40 mL and the product was precipitated as a salt by addition of acetone (300 mL) and left in a refrigerator for a 1h. After filtration, the precipitate was suspended in water (100 mL), rendered basic to pH ~ 12 and stirred for 1h. pH was readjusted to ~8.5, extracted with a 1% of methanol in chloroform (3x100 mL). Evaporation of the combined extracts gave a residue which was chromatographed on basic alumina [elution with a gradient of chloroform:methanol (100:0 to 90:10) followed by a gradient of chloroform:methanol:isopropylamine (95:4:1 to 90:8:2)]. The major fraction was collected (TLC- R<sub>f</sub>, 0.3 in 6% isopropyl amine in chloroform) and evaporated to give **6b-d** as powders.

**5-{3-[4-(3-Amino-propyl)-piperazin-1-yl]-propylamino}-8-methoxy-2,10b-diaza-aceanthrylen-6-one (6b)**

A solution of **5b** (6 g, 0.013 mol) in formic acid was reacted with SnCl<sub>2</sub> in concentrated hydrochloric acid as described in the general procedure above to give 3.5 g (60%) of **6b** as yellow powder: <sup>1</sup>H NMR (CDCl<sub>3</sub>) 9.00 (t, 1H), 8.48 (s, 1H), 8.00-7.91 (m, 2H), 7.84 (d, 1H), 7.36 (dd, 1H), 6.78 (d, 1H), 3.97 (s, 3H), 3.56-3.42 (m, 2H), 2.75 (t, 2H), 2.64-2.44 (m, 10H), 2.42 (t, 2H), 2.06-1.89 (m, 2H), 1.72-1.56 (m, 2H), 1.48 (br s, 2H). Anal. (C<sub>25</sub>H<sub>32</sub>N<sub>6</sub>O<sub>2</sub>·H<sub>2</sub>O) C, H, N.

**5-{3-[4-(3-Amino-propyl)-piperazin-1-yl]-propylamino}-8-methyl-2,10b-diaza-aceanthrylen-6-one (6c)**

Using the general procedure for the synthesis **6b-d**, **5c** (4.7 g 10.39 mmol) was converted to **6c** (2.79, 62%) as a yellow solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>) 9.01 (t, 1H), 8.51 (s, 1H), 8.32 (d, 1H), 7.95 (d, 1H), 7.81 (d, 1H), 7.59 (dd, 1H), 6.78 (d, 1H), 3.52-3.46 (m, 2H), 2.75 (t, 2H), 2.65-2.34 (m, 10H), 2.54 (s, 3H), 2.44-2.36 (m, 2H), 2.20-1.91 (m, 2H), 1.70-1.59 (m, 2H), 1.37 (br s, 2H). Anal. (C<sub>25</sub>H<sub>32</sub>N<sub>6</sub>O·0.3H<sub>2</sub>O) C, H, N.

**5-{3-[4-(3-Amino-propyl)-piperazin-1-yl]-propylamino}-7-methyl-2,10b-diaza-aceanthrylen-6-one (6d)**

Following the general procedure for the synthesis **6b-d**, a yellow powder of **6d** (2.61 g 58%) was obtained from **5d** (4.7 g, 10.39 mmol). <sup>1</sup>H NMR (CDCl<sub>3</sub>) 9.10 (t, 1H), 8.49 (s, 1H), 7.89 (d, 1H), 7.74 (d, 1H), 7.58 (dd, 1H), 7.21 (d, 1H), 6.76 (d, 1H), 3.54-3.38 (m, 2H), 3.01 (s, 3H), 2.75 (t, 2H), 2.68-2.28 (m, 12H), 2.06-1.51 (m 6H). Anal. (C<sub>25</sub>H<sub>32</sub>N<sub>6</sub>O·TFA) C, H, N.

## General procedure for the preparation of 7a-h

An equimolar mixture of an appropriate naphthalic anhydride and substituted 5-amino-2,10b-diazaaceanthrylen-6-one (**6**) was stirred at 80°C in dry DMF (8 mL) under Argon until the reaction was completed (TLC). After cooling the reaction mixture to room temperature, methanol (30 mL) was added and the precipitated solid was filtered, washed with methanol and dried. The resulting orange solid was purified by column chromatography (a gradient of 1-5% of methanol in chloroform as eluent) on basic alumina. Further purification was done using preparative reverse phase HPLC. The dry trifluoroacetate salts thus obtained were suspended in water, neutralized with aqueous sodium bicarbonate solution, filtered, washed with water and dried to give a powder of **7**.

### 2-(3-{4-[3-(8-Fluoro-6-oxo-6H-2,10b-diazaaceanthrylen-5-ylamino)-propyl]piperazin-1-yl}propyl)-5-nitro-2-aza-phenalene-1,3-dione (**7a**)

**6a** (1.54 g, 3.53 mmol) was reacted with 3-nitro-1,8-naphthalic anhydride (0.858 g, 3.53 mmol) and purified as detailed above to yield an orange powder of **7a** (1.958 g, 84%). mp 238-240 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>) 9.31 (d, 1H), 9.13 (d, 1H), 8.96 (t, 1H), 8.78 (m, 1H), 8.51 (s, 1H), 8.43 (m, 1H), 8.22 (m, 1H), 7.97 (d, 1H), 7.93 (m, 2H), 7.53 (m, 1H), 6.79 (d, 1H), 4.29 (m, 2H), 3.47 (qt, 2H), 2.53 (t, 2H), 2.48 (br m, 4H), 2.39 (t, 2H), 2.34 (br m, 4H), 1.96 (m, 2H), 1.88 (m, 2H). Anal. (C<sub>36</sub>H<sub>32</sub>N<sub>7</sub>O<sub>5</sub>F) C, H, N.

### 2-(3-{4-[3-(8-Methoxy-6-Oxo-6H-2,10b-diazaaceanthrylen-5-ylamino)propyl]piperazin-1-yl}propyl)-5-nitro-2-aza-phenalene-1,3-dione (**7b**)

General procedure for the preparation of **7** was followed to obtain **7b** from a mixture of 3-nitro-1,8-naphthalic anhydride (0.869 g, 3.57 mmol) and **6b** (1.6 g, 3.57 mmol). Pure **7b** was obtained as an orange solid (2.18 g, yield 91%), <sup>1</sup>H NMR (CD<sub>3</sub>COOD), 9.20 (d, 1H), 9.14 (d, 1H), 9.00 (s, 1H), 8.73 (dd 1H), 8.55 (m, 1H), 8.11 (d, 1H), 8.03 (d, 1H), 7.97 (dd, 1H), 7.85 (d, 1H), 7.45 (dd, 1H), 6.86 (d, 1H), 4.27 (t, 2H), 3.96 (s, 3H), 3.78-3.62 (br m, 8H), 3.59 (t, 2H), 3.42-3.30 (m, 4H), 2.29-2.20 (br m, 4H). Anal. (C<sub>37</sub>H<sub>35</sub>N<sub>7</sub>O<sub>6</sub>.1/2H<sub>2</sub>O) C, H, N.

### 2-(3-{4-[3-(8-Methyl-6-Oxo-6H-2,10b-diazaaceanthrylen-5-ylamino)propyl]piperazin-1-yl}propyl)-5-nitro-2-aza-phenalene-1,3-dione (**7c**)

Following the general procedure described above for the synthesis of **7**, 3-nitro-1,8-naphthalic anhydride (0.212 g, 0.87 mmol) was reacted with **6c** (0.38 g, 0.87 mmol) to afford an orange solid of **7c** (0.509 g, 89%): <sup>1</sup>H NMR (CF<sub>3</sub>COOD) 9.83 (s, 1H), 9.47 (d, 1H), 9.39 (d, 1H), 8.94 (d, 1H), 8.68 (d, 1H), 8.42 (s, 1H), 8.24 (d, 1H), 8.17 (d, 1H), 8.12 (t, 1H), 7.96 (d,

1H), 7.34 (d, 1H), 4.52 (t, 2H), 4.34-4.19 (m 4H), 4.02-3.66 (m 10H), 2.70 (s, 3H), 2.62-2.46 (m, 4H). Anal. (C<sub>37</sub>H<sub>35</sub>N<sub>7</sub>O<sub>5</sub>·2TFA·3H<sub>2</sub>O)

**2-(3-{4-[3-(7-Methyl-6-Oxo-6H-2,10b-diazaaceanthrylen-5-ylamino)propyl]piperazin-1-yl}propyl)-5-nitro-2-aza-phenalene-1,3-dione (7d)**

**6d** (0.16 g, 0.37 mmol) was treated with 3-nitro-1,8-naphthalic anhydride (0.09 g, 0.37 mmol) according to the general procedure for the synthesis of **7** to give **7d** (0.226 g, 93%) as an orange powder: <sup>1</sup>H NMR (CF<sub>3</sub>COOD) 9.78 (s, 1H), 9.46 (d, 1H), 9.39 (d, 1H), 8.94 (d, 1H), 8.68 (d, 1H), 8.18-8.06 (m, 3H), 7.91 (dd, 1H), 7.71 (d, 1H), 7.33 (d, 1H), 4.59-4.45 (m, 2H), 4.37-4.15 (m, 4H), 4.04-3.62 (m, 10H), 3.09 (s, 3H), 2.62-2.41 (m, 4H). Anal. (C<sub>37</sub>H<sub>35</sub>N<sub>7</sub>O<sub>5</sub>·3TFA) C, H, N.

**2-(3-{4-[3-(6-Oxo-6H-2,10b-diazaaceanthrylen-5-ylamino)propyl]piperazin-1-yl}propyl)-2-aza-phenalene-1,3-dione (7e)**

A light orange solid of **7e** (0.162 g, 64%) was obtained from a mixture of **6e** (0.072 g, 0.172 mmol) with 1,8-naphthalic anhydride (0.034 g, 0.172 mmol) using the general procedure for the synthesis of **7**: <sup>1</sup>H NMR (CF<sub>3</sub>COOD) 9.89 (s, 1H), 8.76 (d, 2H), 8.63 (d, 1H), 8.48 (d, 2H), 8.36 (d, 1H), 8.19 (d, 1H), 8.16-8.09 (m, 1H), 8.00-7.89 (m, 3H), 7.35 (d, 1H), 4.54 (dd, 2H), 4.36-4.1 (m, 4H), 4.02-3.64 (m, 10H), 2.62-2.47 (m, 4H). Anal. (C<sub>36</sub>H<sub>34</sub>N<sub>6</sub>O<sub>3</sub>·3TFA·3H<sub>2</sub>O) C, H, N.

**2-(3-{4-[3-(8-Methoxy-6-Oxo-6H-2,10b-diazaaceanthrylen-5-ylamino)propyl]piperazin-1-yl}propyl)-2-aza-phenalene-1,3-dione (7f)**

Using the general procedure for the preparation of **7**, **6b** (0.101 g, 0.226 mmol) was condensed with 1,8-naphthalic anhydride (0.045 g, 0.226 mmol) to afford **7f** (0.109 g, 77%) as an orange powder: <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.98 (t, 1H), 8.59 (d, 1H), 8.58 (d, 1H), 8.48 (s, 1H), 8.19 (d, 1H), 8.18 (d, 1H), 7.97-7.92 (m, 2H), 7.83 (d, 1H), 7.75 (d, 1H), 7.73 (d, 1H), 7.36 (dd, 1H), 6.75 (d, 1H), 4.26 (t, 2H), 3.97 (s, 3H), 3.49-3.41 (m, 2H), 2.64-2.31 (m, 12H), 2.02-1.86 (m, 4H). Anal. (C<sub>37</sub>H<sub>36</sub>N<sub>7</sub>O<sub>4</sub>·3TFA·H<sub>2</sub>O) C, H, N.

**2-(3-{4-[3-(6-Oxo-6H-2,10b-diazaaceanthrylen-5-ylamino)propyl]piperazin-1-yl}propyl)-6-nitro-2-aza-phenalene-1,3-dione (7g)**

Preparation of **7g** (0.065 g, 59%) was carried out from a mixture of **6e** (0.071 g, 0.17 mmol) and 4-nitro-1,8-naphthalic anhydride (0.041 g, 0.17 mmol) using the general procedure described above: <sup>1</sup>H NMR (CF<sub>3</sub>COOD) 9.90 (s, 1H), 9.03 (d, 1H), 8.91-8.82 (m, 2H), 8.63 (d, 1H), 8.56 (d, 1H), 8.37 (d, 1H), 8.22-8.09 (m, 3H), 7.99-7.92 (m, 1H), 7.35 (d, 1H), 4.51

(t, 2H), 4.34-4.18 (m, 4H), 4.01-3.63 (m, 10H), 2.63-2.46 (m, 4H). Anal. (C<sub>36</sub>H<sub>33</sub>N<sub>7</sub>O<sub>5</sub>·2TFA·2H<sub>2</sub>O) C, H, N.

**2-(3-{4-[3-(8-Methoxy-6-Oxo-6*H*-2,10b-diazaaceanthrylen-5-ylamino)propyl]piperazin-1-yl}propyl)-6-nitro-2-aza-phenalene-1,3-dione (7h)**

Condensation of **6b** (0.065 g, 0.15 mmol) and 4-nitro-1,8-naphthalic anhydride (0.035 g, 0.15 mmol) according to the procedure described for **7** led to the formation of **7h** (0.088 g, 90%) as an orange powder: <sup>1</sup>H NMR (CF<sub>3</sub>COOD) 9.81 (s, 1H), 9.03 (d, 1H), 8.90-8.83 (m, 2H), 8.56 (d, 1H), 8.32 (d, 1H), 8.22-8.13 (m, 2H), 8.07 (d, 1H), 7.73 (dd, 1H), 7.34 (d, 1H), 4.51 (t, 2H), 4.27 (t, 4H), 4.14 (s, 3H), 4.02-3.65 (m, 10H), 2.63-2.46 (br m, 4H). Anal. (C<sub>37</sub>H<sub>35</sub>N<sub>7</sub>O<sub>6</sub>·2TFA·2H<sub>2</sub>O) C, H, N.

**5-Amino-2-(3-{4-[3-(6-oxo-6*H*-2,10b-diaza-aceanthrylen-5-ylamino)propyl]piperazin-1-yl}-propyl)-2-aza-phenalene-1,3-dione (7i)**

Stannous chloride (1.52 g, 0.008 mol) dissolved in concentrated hydrochloric acid (5 mL) was added to a stirred solution of **7a** (0.661 g, 0.001 mol) in glacial acetic acid (25 mL). The mixture was stirred at 60 °C for 2 h. After cooling, acetone (50 mL) was added and the mixture was stirred vigorously. Precipitate was collected by filtration, washed with acetone, and suspended in water (250 mL). The suspension was made basic (pH~12) with sodium hydroxide and the product was extracted with chloroform (5 x 50 mL). Crude product was chromatographed on silica gel column with chloroform-methanol (10:1) mixture containing 0.5% isopropylamine. The main fraction, after evaporation of solvents, yielded 0.397 g (63%) of yellow solid of **7i**: mp 240-243°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>) 8.78 (m, 1H), 8.51 (s, 1H), 8.31 (m, 1H), 8.22 (m, 1H), 8.02 (d, 1H), 7.97 (m, 1H), 7.92 (m, 2H), 7.60 (m, 1H), 7.29 (d, 1H), 6.79 (d, 1H), 4.24 (m, 2H), 4.16 (s, 2H), 3.46 (qt, 2H), 2.51 (t, 2H), 2.48 (br m, 8H), 2.43 (t, 2H), 2.34 (br m, 4H), 1.94 (m, 2H), 1.88 (m, 2H). Anal. (C<sub>36</sub>H<sub>34</sub>N<sub>7</sub>O<sub>3</sub>·1/2H<sub>2</sub>O) C, H, N.

**Preparation of the salts for biological testing:**

To a stirred suspension of **7** (~0.75 mmol) in water (~60 mL) was added methane sulfonic acid (2.25 mmol). The compound was dissolved upon sonication. The resulting solution was lyophilized to give hygroscopic orange-yellow salt. Salts were assumed to be 1:3 (free base:methane sulfonic acid) ratio. These salts were dissolved in water to make solutions of required concentrations for *in vitro* and in 5% dextrose for *in vivo* studies.

**Table S1. Analytical data for compounds described <sup>a</sup>.**

	%C Theory	%C, Found	%N, Theory	%N, Found	%H, Theory	%H, Found	Formula	Mass, Theory	Mass, Found <sup>b</sup>
<b>7a</b>	65.35	65.39	14.82	14.92	4.87	4.88	C <sub>36</sub> H <sub>32</sub> FN <sub>7</sub> O <sub>5</sub>	661.24	661.3±0.1
<b>7b</b>	65.09	65.01	14.36	14.33	5.31	5.20	C <sub>37</sub> H <sub>35</sub> N <sub>7</sub> O <sub>6.1/2</sub> H <sub>2</sub> O	673.26	673.2±0.1
<b>7c</b>	52.40	52.52	10.43	10.31	4.61	3.53	C <sub>37</sub> H <sub>35</sub> N <sub>7</sub> O <sub>5.2</sub> TFA.3H <sub>2</sub> O	657.27	657.3±0.1
<b>7d</b>	51.66	51.86	9.81	9.79	3.83	3.64	C <sub>37</sub> H <sub>35</sub> N <sub>7</sub> O <sub>5.3</sub> TFA	657.27	657.3±0.1
<b>7e</b>	50.71	50.79	8.45	8.68	4.36	3.62	C <sub>36</sub> H <sub>34</sub> N <sub>6</sub> O <sub>3.3</sub> TFA.3H <sub>2</sub> O	598.27	598.3±0.1
<b>7f</b>	52.23	52.12	8.50	8.30	4.18	4.15	C <sub>37</sub> H <sub>36</sub> N <sub>7</sub> O <sub>4.3</sub> TFA.H <sub>2</sub> O	628.28	628.3±0.1
<b>7g</b>	52.92	52.70	10.80	10.38	4.33	3.68	C <sub>36</sub> H <sub>33</sub> N <sub>7</sub> O <sub>5.2</sub> TFA.2H <sub>2</sub> O	643.25	643.3±0.1
<b>7h</b>	52.51	52.23	10.46	10.11	4.41	4.07	C <sub>37</sub> H <sub>35</sub> N <sub>7</sub> O <sub>6.2</sub> TFA.2H <sub>2</sub> O	673.26	673.3±0.1
<b>7i</b>	67.49	67.68	15.30	15.41	5.51	5.47	C <sub>36</sub> H <sub>34</sub> FN <sub>7</sub> O <sub>3.1/2</sub> H <sub>2</sub> O	631.27	631.3±0.1

<sup>a</sup> Data for 5d, 6d and 7c-h were obtained for their TFA salts.

<sup>b</sup> The molecular mass was determined by ion-spray mass spectrometry utilizing Agilent1100 LC/MS system.

**Chemicals.** All mammalian cell culture reagents were purchased from GIBCO-BRL (Grand Island, N.Y). Tissue culture materials were from Corning Incorporated (Acton, MA).

Propidium Iodide (PI), RnaseA and ATP (Adenosine 5'- triphosphate disodium salt), pBR322 plasmid DNA were from Sigma-Aldrich, St.Louis, MO.). DNA Typing Grade Agarose, TBE (Tris-Boric Acid/EDTA) and TAE (Tris-Acetate/EDTA) buffers, Ethidium Bromide, and Trypan Blue Stain were purchased from GIBCO-BRL (Grand Island, N.Y).

Topoisomerase I was from Invitrogen( ), Human type II Topoisomerase and Topoisomerase II assay kit were from TopoGen, Inc. (Columbus, Ohio).

**Cell Lines and Culture Conditions.** Human colon carcinoma HCT116 (p53 wt), RKO (p53 wt), HT29 (p53 mt) and COLO205 (p53 mt), human leukemia HL60 (p53 null) cells were purchased from the American Type Culture Collection (Rockville, MD). HCT116, RKO, and HT29 cells were grown in Dulbecco's Modified Eagle Medium (DMEM), COLO205 and

HL60 cells in RPMI1640 medium. The media were supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100µg/ml streptomycin.

The human pancreatic carcinoma 10.05 (p53 mt) cell line was established and generously provided by Dr. Elisabeth Jaffe of Johns Hopkins University (3), ASPC-1 (p53-mt) cell line was purchased from the American Type Cell Culture. The cells were grown in RPMI 1640 medium supplemented with 15% FBS, 10mM MEM nonessential amino acids, 100mM MEM sodium pyruvate, 10mM HEPES buffer, 0.2 unit/ml insulin, 2mM L-glutamine and standard antibiotics.

Cultures were incubated at 37<sup>0</sup>C in a 5% CO<sub>2</sub> humidified atmosphere.

**Cytotoxicity Assay.** Cells were seeded in six wells for each studied concentration into 96-well microtiter plates (100 µl of medium containing 1000-3000 cells per well) and were precultured for 1 day. Stock solutions (5 mM and 0.5 mM) of test compounds were prepared freshly by dissolving their salt forms in distilled water. These solutions were used to prepare 2 µM of working solution and its 10-fold serial dilutions in appropriate media. 100 µl of drug containing medium or vehicle (control) was added to each well. The cytotoxicity was determined by the MTT-based, CellTiter96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to instructions provided by the supplier of the kit. While the drugs were added, assays were performed on extra reference plates to determine the cell population density at time 0 (T<sub>0</sub>). After 120 h of incubation at 37 °C in a humidified atmosphere containing 5 % CO<sub>2</sub>, the assays were performed on test (T) and control (C) cells. The absorbance of the wells was determined at 544 nm by FLUOstar/POLARstar Galaxy (BMG Labtechnologies GmbH) Microplate Reader. Cellular responses were calculated from the data as described previously (23):  $100 \times [(T-T_0)/(C-T_0)]$  for T>T<sub>0</sub> and  $100 \times [(T-T_0)/T_0]$  for T<T<sub>0</sub>.

**Flow Cytometry Analysis.** Tumor cells in the exponential phase of growth were seeded at a density of 0.5-1 x 10<sup>6</sup> cells in 25 cm<sup>2</sup> or 75cm<sup>2</sup> T flasks and allowed to attach for 24 hours. Then they were exposed to 50 or 100nM of WMC-79 or HKH40A. At appropriate intervals, treated and control cells were harvested by trypsinization, combined with the removed medium that might contain floating cells, and centrifuged at 4°C. Cell pellets were washed twice in ice-cold PBS containing 1% fetal bovine serum. The cells were fixed in 70% ethanol, stored at -20<sup>0</sup>C until all time points had been collected and stained for fluorescence-activated cell sorting (FACS) according to standard procedures (4). Briefly, fixed cells were rinsed twice in ice-cold PBS containing 10% fetal bovine serum then they were treated with RNase A (1 unit/10<sup>6</sup> cells) for 30 min at 37<sup>0</sup>C. Samples chilled in ice were stained overnight in the cold and dark, with propidium iodide (50µg/ml). Cell cycle analysis was performed using Coulter fluorescence activated cell analyzer with 10000 events per determination. The data was analyzed by a multicycle program provided by the manufacturer. The percentage of cells in subG<sub>0</sub>/G<sub>1</sub>, G<sub>1</sub>, S and G<sub>2</sub>/M were also calculated.

**Table S2.** NCI 60 cell line toxicity data for 7b (NSC D-725785)

National Cancer Institute Developmental Therapeutics Program		NSC : D - 725785 / 3		Units : Molar		SSPL : 272F		EXP. ID : 0506NS78	
Mean Graphs									
Report Date : May 16, 2007									
Test Date : June 06, 2005									
Panel/Cell Line	Log <sub>10</sub> GI50	GI50	Log <sub>10</sub> TGI	TGI	Log <sub>10</sub> LC50	LC50			
<b>Leukemia</b>									
CORF-CEM	-9.39		-7.01		> -6.00				
HL-60(TB)	-9.80				> -6.00				
K-562	-8.76		-8.73		> -6.00				
RFMI-8226	-8.39		> -6.00		> -6.00				
SR	< -10.00		< -10.00		> -7.77				
<b>Non-Small Cell Lung Cancer</b>									
EVX	-8.48		-7.23		> -6.00				
HOP-92	-8.29		-7.20		> -6.00				
NCI-H226	-8.88		-7.49		> -6.00				
NCI-H23	-7.69		> -6.00		> -6.00				
NCI-H322M	-8.23		-8.51		> -6.00				
NCI-H460	-8.59		> -6.00		> -6.00				
NCI-H522	-8.38		-8.82		> -6.00				
<b>Colon Cancer</b>									
COLO 205	-8.20		> -6.00		> -6.00				
HCC-2998	-7.94		> -6.00		> -6.00				
HCT-116	-9.39		-7.82		> -6.67				
HCT-15	-8.51		> -6.00		> -6.00				
HT29	-8.56		-8.53		> -6.00				
KM12	-8.36		> -6.27		> -6.00				
SW-620	-9.17		> -6.00		> -6.00				
<b>CNS Cancer</b>									
SF-298	-7.99		> -6.00		> -6.00				
SF-295	-9.00		-7.10		> -6.00				
SF-830	-8.56		-7.60		> -6.00				
SNB-19	-8.07		> -6.00		> -6.00				
SNB-75	-8.17		-6.67		> -6.00				
U251	-8.66		> -6.00		> -6.00				
<b>Melanoma</b>									
LOX IMVI	-8.73		-7.11		> -6.00				
MALME-3M	-8.22		-7.02		> -6.00				
SK-MEL-2	-7.56		> -6.00		> -6.00				
SK-MEL-28	-8.26		-8.03		> -6.00				
SK-MEL-5	-8.31		-7.36		> -6.06				
UACC-62	-8.48		-7.26		> -6.00				
<b>Ovarian Cancer</b>									
IGROV1	-8.47		-7.29		> -6.00				
OVCAR-3	-7.30		> -6.00		> -6.00				
OVCAR-4	-8.31		-7.06		> -6.00				
OVCAR-5	-6.47		> -6.00		> -6.00				
SK-OV-3	-6.82		> -6.00		> -6.00				
<b>Renal Cancer</b>									
786-D	-8.29		> -6.00		> -6.00				
A-498	-8.18		-6.48		> -6.00				
ACHN	-9.39		-7.20		> -6.00				
CAKI-1	-8.52		-7.39		> -6.00				
SN12C	-8.60		> -6.00		> -6.00				
TK-10	-7.87		> -6.00		> -6.00				
UO-31	-8.57		-7.75		> -6.00				
<b>Prostate Cancer</b>									
PC-3	-8.51		> -6.47		> -6.00				
DU-145	-8.43		> -6.00		> -6.00				
<b>Breast Cancer</b>									
MCF7	-8.33		> -6.00		> -6.00				
NCI/ADR-RES	-7.62		> -6.00		> -6.00				
MDA-MB-231/ATCC	-8.32		> -6.00		> -6.00				
HS 578T	-7.83		> -6.00		> -6.00				
MDA-MB-435	-8.13		> -6.00		> -6.00				
BT-549	-8.51		-7.27		> -6.00				
T-47D	-8.06		> -6.00		> -6.00				
<b>MID</b>									
Delta	-8.36		-6.6		-6.05				
Range	1.64		3.4		1.72				
	3.53		4.0		1.77				

**Protocol for pharmacokinetics studies.**

Specific HPLC assays were developed for the analysis of **1** and **7b** concentrations in plasma. Chromatography was performed using an Agilent 1100 series system operated at a flow rate of 1 ml/min. For assay of **1**, separations were conducted under isocratic reversed-phase conditions on a 150 mm x 4.6 mm I.D. FluoroSep RP (ES Industries, West Berlin, NJ) column, eluted with a mobile phase composed of acetonitrile-50 mM, pH 3 ammonium formate (67:33, v/v). UV absorption of the column effluent was monitored at a wavelength of 280 nm. For assay of **7b**, separations were conducted under gradient reversed-phase conditions on a 150 mm x 4.6 mm I.D. SymmetryShield RP8 (Waters, Inc., Milford, MA) column, eluted with 1% acetic acid containing incrementally increasing proportions of acetonitrile (0-100%) over 11 minutes. UV absorption of the column effluent was monitored at a wavelength of 290 nm.

For murine studies, male, young adult CD2F1 (average body weight approximately 28 g) or ncr nu/nu (average body weight approximately 25 g) mice (Harlan Sprague–Dawley, Frederick, MD) were employed. Rat studies were conducted in male, F344/Ntac rats (average body weight 285g) with catheters surgically implanted in the jugular vein (Taconic, Inc., Germantown, NY). Housing, animal care and all experimental procedures and manipulations were carried out in strict compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the NCI-Frederick Animal Care and Use Committee.

For pilot studies, **1** or **7b** was administered to CD2F1 mice at a dose of 20 mg/kg by i.v. (tail vein) or i.p. injection, or by intragastric intubation. The compounds were dissolved in 40%  $\beta$ -hydroxypropyl cyclodextrin (Pharmaceutical Resources Branch, Developmental Therapeutics Program, NCI), and given in a dose volume of 2 ml/kg. Blood was collected from groups of 3 mice 5, 15, 60 and 120 minutes following compound administration, and processed as described<sup>2</sup>. For all other murine studies, **7b** was given to nude mice at a dose of 20mg/kg by i.v. (tail vein) or s.c. (inter-scapular) injection. The compound was dissolved in 5% dextrose and administered in a dose volume of 4 mL/kg body weight. At selected intervals from 3 (i.v.) or 5 (s.c.) minutes through 24 hours, blood was collected from groups of 3 mice, and samples were processed as described<sup>2</sup>. For rat studies, **7b** was given to single rats at a dose of 20 mg/kg by i.v. (jugular vein catheter) or i.p. injection, or by intragastric intubation. The compound was dissolved in 5% dextrose and administered in a dose volume of 1 ml/kg body weight. At selected intervals from 3 minutes through 4 hours for the i.v. study, or 5 minutes through 24 hours for the i.p. and p.o. studies, blood was collected *via* a jugular vein catheter, and samples were processed as described<sup>2</sup>.

Plasma samples (50 $\mu$ l) were prepared for analysis by precipitation of plasma proteins with acetonitrile (200 $\mu$ l). Following centrifugation at 13,000g for 10 min, the supernatant was either evaporated then reconstituted in 200  $\mu$ l distilled water (assay of **1**), or 200 $\mu$ l were mixed with 100 $\mu$ l of 1% acetic acid (assay of **7b**), and plasma concentrations of **1** or **7b** were determined by HPLC as described above. Plasma concentration versus time profiles were constructed using the geometric mean of the plasma concentrations for the individual animals at each time point, and the mean of the times of collection. The line of best fit was determined by nonlinear regression analysis, and pharmacokinetic parameters were calculated by standard equations as previously described<sup>2</sup>.

### **Protocol for *in-vivo* studies**

Female nude mice (nu/nu) between 5 and 6 weeks of age weighing approximately 20 grams were obtained from Harlan, Inc. (Wisconsin, WI). Mia PaCa-2 pancreatic tumor cell line and HCT 116 colon tumor cell line were obtained from American Type Culture Collection. Animals were implanted subcutaneously by trocar with fragments of Mia PaCa-2 or HCT 116 tumor fragments harvested from SC growing tumors in host nude mice. When tumors reached approximately 62 mg in weight for HCT116 cells (13 days following implantation) or 82 mg for Mia Paca-2 cells (29 days following implantation) animals were pair-matched by tumor size into treatment and control groups. **7b** in vehicle (5% dextrose in sterile water)

was administered by IV injections. Tumor measurements were taken by manual caliper twice weekly, starting on day 1.

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