

**Improving rescinamine as an inducer of MSH2-dependent apoptosis in cancer
treatment**

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We, and others have previously shown that mismatch repair proteins, in addition to their repair function, have the ability to contribute to cell death initiation. In response to some drugs, this cell death activity is independent of the repair function of the proteins. Rescinnamine, a derivative of the indole alkaloid reserpine, a drug used to treat hypertension several decades ago, was shown to target the cell death-initiating activity of mismatch repair proteins. When used in animals, the hypotensive action of this drug prevents applying appropriate concentrations for statistically significant tumor reduction. Here we used a combination of computational modeling, chemical synthesis and cell assays to determine how rescinnamine can be modified and what effect these modifications have on cell survival. Results inform further computational modeling to suggest new lead molecules to move toward further testing.

a) Introduction

Mismatch repair proteins function in a number of cellular processes, of which cytotoxic response and apoptosis is one of them. The precise mechanism of their involvement in apoptosis remains to be determined and appears to be dependent on the type of damage to the cell (REF). We previously used computational modeling to identify a distinct, death-inducing conformation of the mismatch repair proteins MSH2/MSH6 (Salsbury et al., 2006) that can be targeted for the treatment of cancer cells (Vasilyeva et al., 2009, 2010). The MSH2/MSH6-dependent cell death pathway activates pro-apoptotic proteins, such as caspase-3 and caspase-9, but is independent of many proteins commonly mutated in cancer, e.g. p53 (Topping et al., 2009).

Our initial computational approach that examined hundreds of possible compounds targeting the MSH2/MSH6 death conformation identified two Rauwolfia alkaloids, reserpine and rescinnamine that are expected to mimic the response induced by cisplatin (Vasilyeva et al., 2009). Rescinnamine showed the best cytotoxic activity by activating pro-apoptotic proteins (Vasilyeva et al., 2010). It is a member of an understudied group of compounds previously used as anti-hypertensive drugs in the 1950s and 60s that target the angiotensin-converting enzyme (ACE). Rescinnamine has lower neurotoxicity, but also lower antihypertensive efficacy than its predecessor, reserpine (Fife et al., 1960). The drug has no known genotoxic or chromosome destabilizing effects (Brambilla & Martinelli, 2006). Neither one of these compounds had been systematically studied as anticancer agents.

We here demonstrate that the anti-hypertensive effects limit the usefulness of rescinnamine as a tumor-inhibiting drug, and employ computational simulation and organic synthesis to modify the compound toward the development of a more efficient anti-cancer drug. Application of the palladium-mediated Heck coupling reaction to this structurally complicated alkaloid natural product allows for the efficient preparation of numerous rescinnamine derivatives to test computational predictions and build a structure-activity relationship (SAR). Computationally, we continued our successful protocol of using structures obtained from molecular dynamics simulations along with docking into structures representative of the death-inducing conformations (Vasilyeva et al., 2009). However, we improved upon our previous methods in three ways. First, we used structures obtained via cluster analysis of a longer-time scale simulation of hMSH2/6 (Negureanu and Salsbury, 2012). Second, we upgraded to Autodock4 (Morris et al, 2009), with its improved force field, from Autodock3 (Morris et al, 1998). Third, we focused on modification of reserpine and

rescinnamine, rather than docking of new compounds. This latter required development of a library of modified compounds, which were then docked and ranked.

b) Materials and Methods

Cell Biology. HEC59 cells (*msh2* deficient) and the paired cell line HEC59 chr. that restores the *MSH2*-deficiency via chromosome transfer have been extensively characterized (Umar et al., 1997). Cells were grown in standard growth media (DMEM-F12 + 10% FBS). Cells were plated in microtiter plates at an appropriate concentration in 100 μ l media and incubated overnight. Media was replaced with media containing drug and allowed to incubate for 24 hours at indicated concentrations. Untreated cells received fresh media with vehicle only. One solution reagent (CellTiter 96)(r) Aqueous One Solution) was added to existing media (20 μ l/well) and allowed to incubate 3-4 hrs. A plate reader was used to record the absorbance at 490 nm. Assays were performed at least in triplicates. Cell viability at each concentration was analyzed for IC₅₀ values using GraphPad Prism 4TM. Graphs represent mean values and standard deviations.

Xenograft. SW416 or HEC59 cells in PBS mixed with Matrigel (1:1; BD Biosciences) were subcutaneously injected into the flank of nude donor mice. Tumors were grown for up to 3 weeks. Mice were euthanized, tumors excised, minced into 3 mm pieces, and surgically implanted into the right flank of acceptor mice (10 per group). Isoflurane anesthesia was provided during tumor inoculation. Injection of compounds was started 3 days following tumor implantation to allow recovery from surgery. Compounds were given intraperitoneally, in a volume of 0.5 ml/mouse with a ¼ inch, 23-gauge needle in a 6 ml plastic syringe. Mice were

monitored based on survival and body weight. Tumor size, measured by caliper, and body weight were monitored daily for 57 days, and the prolongation of median survival time after ip treatment determined. Any animals showing signs of distress, unnatural movements, severe loss of appetite, severe signs of hypotension, tumor size of 1000g, or weight loss exceeding 10% before the end of the study were euthanized. Tumors were measured twice a week for each group. Tumor volume is calculated as length (mm) x width (mm)². Initial measurements were performed when the tumor reached 150-200 mg. Tumor weight (in mg) is calculated as tumor weight (mg) - (length(mm) of tumor x width (mm) of tumor²)/2.

Chemistry-General. Reagents were obtained from commercial sources and used without additional purification. Extraction and flash chromatography solvents were technical grade. Flash chromatography was carried out using a Biotage SP1-B2A0/ HPFC System. Analytical thin layer chromatography (TLC) was performed as silica gel plates with C-4 Spectroline 254 indicator. Visualization was accomplished with UV light and 20% phosphomolybdic acid solution in EtOH. LC-MS, ESI-MS and HPLC solvents were HPLC grade. Melting points were determined on a Mel-Temp apparatus. ¹H NMR and ¹³CNMR spectra were taken in commercial deuterated solvents and recorded on a Bruker Advance 300 MHz and Bruker DRX-500 spectrometer using a 5mm TBI probe equipped with z axis gradients. Probe temperature was regulated at 25° C. All data was collected and processed with Topspin 1.3 using standard Bruker processing parameters. Chemical shifts (δ) are given in ppm; multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublet) and br (broad).

Acryloyl reserpate (1). Dry distilled pyridine (791mg, 0.808mL, 10mmole) was added to a mixture of methyl reserpate (300mg, 0.74mmole) and acryloyl chloride (135.77mg, 0.123mL, 1.5mmole) was added and stirred under nitrogen at room temperature for 74 hr. Excess pyridine

was evaporated and the residue was taken up in chloroform (50 mL) and the organic layer washed with water (3x 20 mL) and brine, the organic layer was dried with anhydrous Na₂SO₄, filtered and evaporated under reduced pressure to give a residue that was purified by flash chromatography (silica gel, chloroform/methanol, 3%, R_f 0.3) giving a solid rinsed with methanol to afford **1** (120 mg, 35%) as white crystals : mp >260 °C; ¹H-NMR (300 MHz, CDCl₃, δ): 7.80 (s, 1H), 7.31(d, 1H, *J*= 8.52 Hz), 6.81 (s, 1H, *J*= 1.95 Hz), 6.76 (d, 1H, *J*= 8.53 Hz), 6.45(d, 1H, *J*= 17.32 Hz), 6.15 (dd, 1H, *J*= 17.31& 17.30 Hz), 5.87 (d, 1H, *J*= 10.4 Hz), 4.90-4.82 (m, 1H), 4.42 (s, 1H), 3.82 (s, 3H), 3.80 (s, 3H), 3.48 (s, 3H), 3.19-3.14 (m, 2H), 3.02-2.98 (m, 1H), 2.69- 2.60 (m, 2H), 2.49-2.39 (m,2 H), 2.26- 2.17 (m, 2H), 2.04-1.78 (m, 5H); ¹³C-NMR (75 MHz, CDCl₃, δ): 172.77, 165.42, 156.10, 136.31, 130.79, 130.45, 128.63, 122.13, 118.45, 108.92, 107.92, 95.18, 77.69, 77.54, 60.68, 55.76, 53.68, 51.75, 51.72, 51.17, 48.96, 33.93, 32.25, 29.47, 24.17, 16.75; ESI-MS: *m/z*= 469.2 (M⁺ + H); Anal. Calcd. for C₂₆H₃₂N₂O₆·0.3H₂O (474.487) C, 65.81; H, 6.94; N, 5.90. Found: C, 65.60; H, 6.83; N, 6.01.

General procedure for the synthesis of rescinnamine derivatives (2-7) A mixture of the aryl iodide (0.507 mmole), acryloyl reserpate (300 mg, 0.641 mmole), Et₃N (0.06 g, 0.09 mL, 0.645 mmol) and Pd(OAc)₂ (1.23 mg, 0.005mmole) in the presence of tri-*o*-tolylphosphine (4.5 mg, 0.021mmole) in acetonitrile (20 mL) was heated with stirring in a capped sealed glass tube under argon at 90°C for 48hrs. After cooling, the solvent was removed under reduced pressure; the residue was dissolved in chloroform (30 mL) and washed with water (3x15 mL) and brine. The organic layer was dried with anhydrous sodium sulfate, filtered and evaporated under reduced pressure to give a residue that was purified by flash chromatography to afford a solid that was recrystallized as described below:

For 2: (R₁ = COOCH₃, R₂ = OCH₃): White crystals recrystallized from methanol, yield = 180 mg (44%), (chloroform/methanol 3%, R_f = 0.7), mp = 215-217 °C; ¹H NMR (500 MHz, CDCl₃, δ): 8.03 (s, 1H), 7.69 (s, 1H), 7.67-7.66 (m, 2H), 7.35 (d, 1H, *J* = 8.52), 7.02 (d, 1H, *J* = 8.77 Hz), 6.86 (s, 1H), 6.79 (d, 1H, *J* = 8.5 Hz), 6.40 (d, 1H, *J* = 15.98), 4.97-4.92 (m, 1H), 4.47 (s, 1H), 3.97 (s, 3H), 3.95 (s, 3H), 3.86 (s, 3H), 3.84 (s, 3H), 3.54 (s, 3H), 3.19-3.18 (m, 2H), 3.06-3.03 (m, 1H), 2.69-2.66 (m, 1H), 2.52-2.45 (m, 3H), 2.30-2.25 (m, 2H), 1.98-1.80 (m, 5H); ¹³C NMR (125 MHz, CDCl₃, δ): 172.94, 166.41, 166.11, 160.58, 156.03, 143.66, 136.30, 133.49, 131.63, 130.43, 126.48, 122.05, 120.28, 118.55, 116.84, 112.34, 108.94, 107.85, 95.04, 65.93, 60.97, 56.26, 55.76, 53.69, 52.40, 51.76, 51.17, 48.92, 33.92, 32.25, 31.08, 29.62, 24.21, 16.75, 15.33; ESI-MS: *m/z* = 633.4 (M⁺ + H); Anal. Calcd for C₃₅H₄₀N₂O₉ (623.70): C, 66.44; H, 6.37; N, 4.43; Found: C, 66.16; H, 6.46; N, 4.41.

For 3 (R₁ = OCH₃, R₂ = COOH): Yellow crystals recrystallized from diethyl ether, yield = 150 mg (38%), (chloroform/methanol 8%, R_f = 0.1), mp = 228-230 °C; ¹H NMR (300 MHz, DMSO, δ): 10.62 (s, 1H), 7.70 (d, 1H, *J* = 15.97 Hz), 7.58 (d, 1H, *J* = 7.83 Hz), 7.47 (s, 1H), 7.32 (d, 1H, *J* = 7.83 Hz), 7.24 (d, 1H, *J* = 8.55 Hz), 6.82 (s, 1H), 6.79 (d, 1H, *J* = 15.91 Hz), 6.63 (d, 1H, *J* = 8.57 Hz), 4.90-4.82 (m, 1H), 4.53 (s, 1H), 3.88 (s, 3H), 3.79 (s, 3H), 3.75 (s, 3H), 3.43 (s, 3H), 3.17-3.11 (m, 3H), 2.97-2.84 (m, 3H), 2.72-2.66 (m, 1H), 2.24 (d, 1H), 1.87-1.73 (m, 6H); ¹³C NMR (75 MHz, DMSO, δ): 171.81, 170.22, 165.82, 157.10, 155.80, 144.75, 137.20, 136.09, 129.43, 128.36, 121.26, 120.57, 118.97, 118.67, 111.83, 108.99, 105.82, 94.88, 79.40, 77.59, 76.63, 60.55, 55.99, 55.40, 53.47, 52.29, 50.73, 50.06, 48.87, 32.21, 31.29, 28.94, 23.05, 16.00; ESI-MS: *m/z* = 619.3 (M⁺ + H); Anal. Calcd for C₃₄H₃₈N₂O₉·2.5H₂O (663.71): C, 61.53; H, 6.53; N: 4.22; Found: C, 61.41; H, 7.11; N, 4.00.

For 4 (R₁ = COOH, R₂ = OCH₃): Pale yellow solid recrystallized from diethyl ether, yield = 108 mg (27%), (chloroform/methanol 8%, R_f = 0.1), mp = 249-250 °C; ¹H NMR (500 MHz, DMSO, δ): 10.61 (s, 1H), 8.32 (s, 1H), 7.93 (s, 1H), 7.87 (d, 1H, *J* = 8.57 Hz), 7.70 (d, 1H, *J* = 15.96 Hz), 7.24 (d, 1H, *J* = 8.44 Hz), 7.16 (d, 1H, *J* = 8.66 Hz), 6.82 (s, 1H), 6.63 (d, 1H, *J* = 8.34 Hz), 6.55 (d, 1H, *J* = 15.99 Hz), 4.81-4.79 (m, 1H), 4.49 (s, 1H), 3.87 (s, 3H), 3.80 (s, 3H), 3.75 (s, 3H), 3.42 (s, 3H), 3.14-3.13 (m, 3H), 2.94-2.85 (m, 3H), 2.68-2.66 (m, 1H), 2.21-2.16 (m, 1H), 1.99-1.74 (m, 6H); ¹³C NMR (125 MHz, DMSO, δ): 172.07, 168.17, 166.19, 159.75, 155.79, 144.34, 137.13, 132.76, 130.86, 126.43, 123.99, 121.87, 118.53, 116.70, 113.15, 108.81, 106.24, 95.28, 79.59, 77.91, 77.02, 60.64, 56.41, 55.67, 53.97, 52.31, 51.31, 50.96, 48.69, 33.17, 32.12, 29.67, 23.67, 16.57; ESI-MS: *m/z* = 619.3 (M⁺ + H); Anal. Calcd for C₃₄H₃₈N₂O₉·3H₂O (672.72): C, 60.70; H, 6.59; N, 4.16; Found: C, 60.76; H, 6.39; N, 4.07.

For 5 (R₁ = OCH₃, R₂ = OH): White flakes from chloroform, yield = 108 mg (27%), (chloroform/methanol 4%, R_f = 0.3), mp = 259-260 °C; ¹H NMR (300 MHz, DMSO, δ): 10.49 (s, 1H), 9.61 (s, br, 1H), 7.62 (d, 1H, *J* = 15.87 Hz), 7.35 (s, 1H), 7.21 (d, 1H, *J* = 8.48 Hz), 7.14 (d, 1H, *J* = 8.35 Hz), 6.82-6.79 (m, 2H), 6.61 (d, 1H, *J* = 8.49 Hz), 6.50 (d, 1H, *J* = 15.89 Hz), 4.87-4.78 (m, 1H), 4.33 (s, 1H), 3.83 (s, 3H), 3.78 (s, 3H), 3.75 (s, 3H), 3.41 (s, 3H), 3.04-3.01 (m, 2H), 2.87-2.81 (m, 2H), 2.66-2.63 (m, 1H), 2.36-2.31 (m, 2H), 2.19-2.12 (m, 1H), 2.04-2.00 (m, 1H), 1.93-1.69 (m, 5H); ¹³C NMR (75 MHz, DMSO, δ): 172.88, 166.49, 156.23, 148.72, 145.98, 144.79, 136.34, 130.55, 128.02, 122.22, 121.86, 118.55, 116.23, 113.18, 110.59, 109.05, 108.13, 95.20, 77.84, 65.84, 60.77, 55.99, 55.82, 53.71, 51.78, 51.19, 49.06, 34.04, 32.34, 29.69, 24.28, 16.81, 15.26; ESI-MS: *m/z* = 591.2 (M⁺ + H).

For 6 (R₁ = OH, R₂ = OCH₃): Yellow solid recrystallized from diethyl ether, yield = 174 mg (43%), (chloroform/methanol 3%, R_f = 0.3), mp = 180-182 °C; ¹H NMR (300 MHz, CDCl₃, δ):

7.77 (s, 1H), 7.63 (d, 1H, $J= 15.92$ Hz), 7.36 (d, 1H, $J= 8.54$ Hz), 7.32 (d, 1H, $J= 8.54$ Hz), 7.27 (s, 1H), 6.97 (d, 1H, $J= 8.56$ Hz), 6.82 (s, 1H), 6.76 (d, 1H, $J= 8.53$ Hz), 6.33 (d, 1H, $J= 15.92$ Hz), 4.95-4.87 (m, 1H), 4.42 (s, 1H), 3.85 (s, 3H), 3.82 (s, 3H), 3.81 (s, 3H), 3.50 (s, 3H), 3.49-3.44 (m, 2H), 3.18-3.14 (m, 2H), 3.02-2.97 (m, 1H), 2.67-2.62 (dd, 1H), 2.49-2.39 (m, 3H), 2.33 (s, 3H), 2.04-1.78 (m, 5H).; ^{13}C NMR (125 MHz, CDCl_3 , δ): 172.86, 168.88, 166.34, 156.18, 152.97, 143.86, 140.06, 136.38, 130.57, 127.82, 127.50, 122.21, 121.94, 118.51, 116.76, 112.35, 108.98, 107.99, 95.25, 77.87, 65.84, 60.79, 56.02, 55.82, 53.74, 51.79, 51.23, 49.04, 34.03, 32.32, 29.69, 24.25, 20.62, 16.81, 15.27; ESI-MS: $m/z= 6.33.3$ ($\text{M}^+ + \text{H}$); Anal. Calcd for $\text{C}_{35}\text{H}_{40}\text{N}_2\text{O}_9 \cdot 0.25\text{H}_2\text{O}$ (637.20): C, 65.97; H, 6.41; N, 4.40; Found: C, 65.74; H, 6.55; N, 4.49.

For 7 ($\text{R}_1 = \text{OCOCH}_3$, $\text{R}_2 = \text{OCH}_3$): Yellow solid recrystallized from diethyl ether, yield = 174 mg (43%), (chloroform/methanol 3%, $R_f = 0.3$), mp = 180-182 °C; ^1H NMR (300 MHz, CDCl_3 , δ): 7.77 (s, 1H), 7.63 (d, 1H, $J= 15.92$ Hz), 7.36 (d, 1H, $J= 8.54$ Hz), 7.32 (d, 1H, $J= 8.54$ Hz), 7.27 (s, 1H), 6.97 (d, 1H, $J= 8.56$ Hz), 6.82 (s, 1H), 6.76 (d, 1H, $J= 8.53$ Hz), 6.33 (d, 1H, $J= 15.92$ Hz), 4.95-4.87 (m, 1H), 4.42 (s, 1H), 3.85 (s, 3H), 3.82 (s, 3H), 3.81 (s, 3H), 3.50 (s, 3H), 3.49-3.44 (m, 2H), 3.18-3.14 (m, 2H), 3.02-2.97 (m, 1H), 2.67-2.62 (dd, 1H), 2.49-2.39 (m, 3H), 2.33 (s, 3H), 2.04-1.78 (m, 5H).; ^{13}C NMR (125 MHz, CDCl_3 , δ): 172.86, 168.88, 166.34, 156.18, 152.97, 143.86, 140.06, 136.38, 130.57, 127.82, 127.50, 122.21, 121.94, 118.51, 116.76, 112.35, 108.98, 107.99, 95.25, 77.87, 65.84, 60.79, 56.02, 55.82, 53.74, 51.79, 51.23, 49.04, 34.03, 32.32, 29.69, 24.25, 20.62, 16.81, 15.27; ESI-MS: $m/z= 6.33.3$ ($\text{M}^+ + \text{H}$); Anal. Calcd for $\text{C}_{35}\text{H}_{40}\text{N}_2\text{O}_9 \cdot 0.25\text{H}_2\text{O}$ (637.20): C, 65.97; H, 6.41; N, 4.40; Found: C, 65.74; H, 6.55; N, 4.49.

Propionoyl reserpate (8). Using the same procedure for **1** substituting propionyl chloride for acryloyl chloride yields a residue that was purified by flash chromatography (silica gel, chloroform/methanol, 3%, R_f 0.2) to give a solid that was rinsed with methanol/diethylether (1:1)

to afford **8** (317 mg, 56%) as a fine light yellow powder : mp = 258 °C; ¹H-NMR (300 MHz, CDCl₃, δ): 7.60 (br, s, 1H), 7.32 (d, 1H, *J*= 8.54 Hz), 6.82 (s, 1H), 6.76 (d, 1H, *J*= 8.59 Hz), 4.81-4.72 (m, 1H), 4.41 (s, 1H), 3.83 (s, 3H), 3.80 (s, 3H), 3.74-3.71 (m, 1H), 3.49 (s, 3H), 3.24-3.10 (m, 2H), 3.03-2.88 (m, 2H), 2.63-2.58 (m, 1H), 2.50-2.31 (m, 2 H), 2.28-2.12 (m, 4H), 2.03-1.75 (m, 4H), 1.17 (t, 3H, *J*= 1.35, 7.39 Hz); ¹³C-NMR (75 MHz, CDCl₃, δ): 173.79, 172.52, 156.21, 136.42, 130.53, 122.27, 118.52, 109.02, 108.05, 95.34, 77.74, 77.32, 60.61, 55.83, 53.73, 51.78, 51.69, 51.22, 49.05, 34.01, 32.33, 29.55, 28.03, 24.25, 16.80, 9.16.; ESI-MS: *m/z*= 471.3 (M⁺ + H); Anal. Calcd. for C₂₆H₃₄N₂O₆. (470.56) C, 66.36; H, 7.28; N, 5.95. Found: C, 66.08; H, 7.44; N, 5.87.

5-iodo-2-methoxyphenyl acetate (9). 2-Methoxyphenylacetate (28.5 g, 171.87 mmol) was added to a mixture of iodine (17.46 g, 68.75 mmol) and HIO₃ (7.18 g, 40.8 mmol) in glacial acetic acid (190 mL), chloroform (50 mL), water (65 mL) and concentrated sulfuric acid (2 mL) and this mixture was stirred for 24 h at 40°C. Chloroform (50 mL) and water (30mL) were added and the mixture was washed with dilute NaHSO₃ (3X) and water. The organic layer was dried with magnesium sulfate and the organic solvent was removed under vacuum to leave a residue that was recrystallized from ethanol to afford **9** as white crystals (11.7 g, 23%); (chloroform/methanol 2%, R_f, 0.3), m.p = 75 °C; ¹H-NMR (300 MHz, CDCl₃, δ): 7.49-7.46 (dd, 1H *J*= 8.63, 2.15 Hz), 7.34 (d, 1H, *J*= 2.17 Hz), 6.73 (d, 1H, *J*= 8.66 Hz), 3.79 (s, 3H), 2.29 (s, 3H); ¹³C-NMR (75 MHz, CDCl₃, δ): 168.58, 151.37, 140.48, 135.71, 131.62, 114.33, 81.32, 55.98, 20.55; ESI-MS: *m/z*= 292.9 (M⁺); Anal. Calcd. for C₉H₉IO₃.0.5CH₃COOH (322.0964) C, 37.29; H, 3.44. Found: C, 37.92; H, 3.09.

N-hydroxy-5-iodo-2-methoxybenzamide (10). Separate solutions of hydroxylamine hydrochloride (605 mg, 8.60 mmole) in methyl alcohol (75 mL) and potassium hydroxide (1.2 g,

18.81 mmole) in methanol (50 mL) are prepared at the boiling point of the solvent. Both are cooled to 30-40 °C and the one containing alkali was added with shaking to the solution of hydroxylamine; any excessive rise of temperature during the addition is prevented by occasional cooling in an ice bath. After all the alkali has been added, the mixture is allowed to stand in an ice bath for 5 min to ensure complete precipitation of potassium chloride followed by filtration. The filtrate was added to methyl 5-iodo-2-methoxybenzoate (500 mg, 1.72 mmole) and the mixture was heated to reflux for 6 h and cooled to room temperature. The mixture was acidified with glacial acetic acid until the pH was about 6 and concentrated to remove the solvents. The residue was mixed with EtOAc (100 mL) and water (80 mL) was added to get a clear solution. The organic layer was separated, and the aqueous solution was extracted with EtOAc (2 x 50 mL). The combined organic phases were washed with brine, dried over anhydrous sodium sulfate, and concentrated to afford a white solid residue, recrystallized from chloroform to afford **9** as white crystals (362 mg, 73% yield) (Ethyl acetate/ Hexane 1:5, $R_f=0.3$); mp = 157-158°C; $^1\text{H-NMR}$ (300 MHz, DMSO, δ): 10.68 (s, 1H), 9.16 (br, s, 1H), 7.82-7.72 (m, 2H), 7.01-6.93 (d, 1H, $J = 8.62$ Hz), 3.81 (s, 3H); $^{13}\text{C-NMR}$ (75 MHz, DMSO, δ): 161.50, 156.48, 139.93, 137.55, 124.89, 114.64, 82.73, 55.85; ESI-MS: $m/z = 294.0$ ($\text{M}^+ + 1$); Anal. Calcd. for $\text{C}_8\text{H}_8\text{INO}_3$ (293.0585) C, 32.79; H, 2.75; N, 4.78. Found: C, 32.82; H, 2.61; N, 4.79.

***N*-hydroxy-4-iodo-2-methoxybenzamide (11)**. This compound was prepared using the same procedure for **7** with methyl-4-iodo-2-methoxybenzoate as substrate to afford an off-white solid that was recrystallized from ethyl acetate/*n*-hexane to yield **10** as off-white crystals (102 mg, 21%); (Ethyl acetate/ Hexane 1:5, $R_f=0.4$); mp = 105-106°C; $^1\text{H-NMR}$ (300 MHz, CDCl_3 , δ): 10.24 (s, br, 1H), 7.82 (d, 1H, $J = 8.23$ Hz), 7.41 (d, 1H, $J = 8.12$ Hz), 7.28 (s, 1H), 3.95 (s, 3H); $^{13}\text{C-NMR}$ (75 MHz, CDCl_3 , δ): 163.13, 157.03, 132.89, 130.94, 120.77, 118.01, 99.66,

56.52; ESI-MS: $m/z = 294.0$ ($M^+ + 1$); Anal. Calcd. for $C_8H_8INO_3 \cdot 0.1CH_3COOC_2H_5$ (301.8690) C, 33.42; H, 2.94; N, 4.64. Found: C, 33.48; H, 2.76; N, 4.57.

General procedure for synthesis of rescinnamine derivatives (12-15): A similar Heck coupling procedure without a phosphine ligand using the appropriate substituted aryl iodides affords the corresponding substituted rescinnamine derivatives (12-15).

For 12: Yellowish brown solid recrystallized from diethyl ether, yield = 132 mg (35%), (chloroform/methanol 2%, $R_f = 0.2$), mp = 202-203 °C; 1H NMR (300 MHz, $CDCl_3$, δ): 7.66 (s, 1H), 7.63 (d, $J = 15.91$ Hz, 1H), 7.32 (d, $J = 8.54$ Hz, 1H), 6.94-6.90 (m, 2H), 6.82 (s, 1H), 6.74 (d, $J = 8.70$ Hz, 2H), 6.27 (d, $J = 15.88$ Hz, 1H), 4.96-4.87 (m, 1H), 4.44 (s, 1H), 3.88 (s, 3H), 3.83 (s, 3H), 3.81 (s, 3H), 3.51 (s, 3H), 3.42-3.44 (m, 1H), 3.20-3.15 (m, 2H), 3.04-2.89 (m, 2H), 2.73-2.61 (m, 1H), 2.50-2.42 (m, 2H), 2.29-2.17 (m, 2H), 2.05-1.77 (m, 4H), 1.28-1.18 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 172.92, 166.68, 156.23, 149.42, 145.33, 136.54, 136.38, 130.51, 127.47, 122.21, 120.30, 118.53, 115.41, 113.13, 110.18, 109.05, 108.06, 95.23, 77.88, 65.84, 60.75, 55.83, 55.59, 53.75, 51.78, 51.21, 49.08, 34.04, 32.32, 29.74, 24.27, 16.81, 15.26; ESI-MS: $m/z = 590$ ($M^+ + 1$), 295 ($M^+ + 2$). HRMS-ESI $^+$ (m/z): $[M + H]^+$ calcd for $C_{33}H_{40}N_3O_7$, 590.2866; found, 590.2846; Anal. Calcd for $C_{33}H_{39}N_3O_7 \cdot 0.8H_2O$ (604.09): C, 65.61; H, 6.77; N, 6.96; Found: C, 65.27; H, 6.63; N, 6.87.

For 13: Pale yellow flakes recrystallized from diethyl ether, yield = 153 mg (41%), (chloroform/methanol 2%, $R_f = 0.4$), mp = 210-213 °C; 1H NMR (300 MHz, $CDCl_3$, δ): 7.81 (s, br, 1H), 7.61 (d, $J = 15.84$ Hz, 1H), 7.31 (d, $J = 8.54$ Hz, 1H), 7.01 (d, $J = 7.98$ Hz, 1H), 6.96 (s, 1H), 6.82 (s, 1H), 6.75 (d, $J = 8.54$ Hz, 1H), 6.66 (d, $J = 7.97$ Hz, 1H), 6.23 (d, $J = 15.83$ Hz, 1H), 4.95-4.80 (m, 1H), 4.43 (s, br, 1H), 4.14 (s, 1H), 3.88 (s, 3H), 3.82 (s, 3H), 3.80 (s, 3H),

3.50 (s, 3H), 3.18-3.13 (m, 2H), 3.03-2.93 (m, 2H), 2.67-2.58 (m, 1H), 2.49-2.40 (m, 2H), 2.27-2.16 (m, 2H), 1.92-1.77 (m, 5H); ^{13}C NMR (75 MHz, CDCl_3 , δ): 172.99, 166.94, 156.15, 146.97, 145.77, 139.20, 136.36, 130.51, 128.67, 124.60, 123.34, 122.15, 118.52, 114.06, 113.39, 108.98, 107.96, 95.19, 60.77, 55.82, 55.50, 53.73, 51.82, 51.19, 49.03, 41.81, 34.01, 32.30, 30.95, 29.77, 24.24, 16.78, 13.85; ESI-MS: $m/z= 590$ ($\text{M}^+ + 1$), Anal. Calcd for $\text{C}_{33}\text{H}_{39}\text{N}_3\text{O}_7 \cdot 0.6\text{H}_2\text{O}$ (600.49): C, 66.01; H, 6.75; N, 7.00; Found: C, 66.11; H, 6.64; N, 6.71.

For 14: Grayish white solid, yield = 289 mg (70%), recrystallized from benzene, (chloroform/methanol 6%, $R_f= 0.3$), mp = 245-255 °C; ^1H NMR (500 MHz, DMSO, δ): 10.96 (s, 1H), 7.57 (s, 1H), 7.49 (d, 1H, $J= 15.78$ Hz), 7.37-7.34 (m, 2H), 7.23 (s, 1H), 6.86 (s, 1H), 6.71 (d, 1H, $J= 8.49$ Hz), 6.27 (d, 1H, $J= 15.79$ Hz), 5.06 (s, br, 1H), 5.06 (s, 1H), 4.69-4.72 (m, 1H), 3.81 (s, 3H), 3.79 (s, 3H), 3.77 (s, 3H), 3.74-3.68 (m, 2H), 3.65-3.57 (m, 1H), 3.44 (s, 3H), 3.17-3.07 (m, 1H), 2.84-2.99 (m, 1H), 2.85-2.82 (m, 1H), 2.73-2.69 (m, 1H), 2.30-2.08 (m, 5H), 1.94-1.83 (m, 2H); ^{13}C NMR (125 MHz, DMSO, δ): 171.76, 171.24, 165.79, 157.77, 155.92, 149.02, 147.59, 145.74, 137.31, 128.29, 124.66, 122.56, 120.84, 120.64, 118.63, 118.35, 112.76, 112.08, 109.04, 105.34, 94.82, 77.12, 75.66, 60.23, 55.66, 55.25, 54.32, 51.95, 50.16, 31.27, 30.34, 28.34, 22.49, 15.49; ESI-MS: $m/z= 635.2$ ($\text{M}^+ + \text{H}$); HRMS: [$\text{M}^+ + \text{H} - \text{CH}_3$] calcd for $\text{C}_{34}\text{H}_{39}\text{N}_2\text{O}_{10}$, 635.2605; found, 635.2582; Anal. Calcd for $\text{C}_{35}\text{H}_{40}\text{N}_2\text{O}_{10} \cdot 2.5\text{H}_2\text{O}$ (679.711): C, 61.71; H, 6.25; N, 4.23; Found: C, 61.84; H, 5.87; N, 4.28.

For 15: Dark yellow solid, yield = 140 mg (35%), recrystallized from diethyl ether, (chloroform/methanol 5%, $R_f= 0.1$), mp = 229-231 °C; ^1H NMR (300 MHz, DMSO, δ): 10.49 (s, 1H), 8.96 (s, br, 1H), 7.63 (d, 1H, $J= 15.81$ Hz), 7.20 (d, 1H, $J= 8.57$ Hz), 7.06 (s, 2H), 6.80 (s, 1H), 6.62-6.54 (m, 2H), 4.89-4.76 (m, 1H), 4.35 (s, 1H), 3.82 (s, 6H), 3.79 (s, 3H), 3.75 (s, 3H), 3.42 (s, 3H), 3.10-2.97 (m, 3H), 2.89-2.79 (m, 3H), 2.69-2.60 (m, 3H), 2.42-2.14 (m, 5H); ^{13}C

NMR (75 MHz, DMSO, δ): 171.61, 165.98, 155.05, 148.01, 145.61, 138.35, 136.35, 131.20, 128.52, 124.37, 121.65, 117.81, 114.96, 108.01, 106.32, 105.92, 94.77, 77.54, 76.40, 64.87, 60.05, 56.09, 55.18, 53.32, 51.74, 51.05, 50.74, 48.62, 33.19, 32.14, 29.53, 23.46, 16.44, 15.12; ESI-MS: $m/z= 621.3$ ($M^+ + H$).

Molecular Docking

The molecular docking was performed in four phases; structural model generation, ligand library generation, receptor grid generation and finally docking of the ligand library into the receptor grid. Models for the structures were generated using molecular dynamics as in our previous works (Vasilyeva, A et al, 2009; Vasilyeva, A et al, 2010; Salsbury, 2-1-). However, for this work more extensive simulations were used. The details of these simulations are reported elsewhere (Negureanu and Salsbury, 2012). In short, the simulations were four 20ns NPT all-atom simulations based on the human MSH2/6 crystal structure (Morris et al, 2009), with the (1,2)G cross-link, which is the predominate damage due to cisplatin. The structure selected was the median structure of the most populated cluster found from all-atom RMSD-based clustering (Negureanu and Salsbury, 2012). The appropriate pdbqs file was generated with the DNA removed, so that just the protein remained, using defaults from Autodock4.

Libraries for docking were based on the core of the rescinnamine structure (Figure 7). All possible derivatives were made based on this structure (Figure 7), with R1, R2 and R3 selected from H, OH, COOH, NH₂, and OCH₃. Also, a structure without the modified phenyl ring was docked (compound 1, Table I). The best of these compounds were suggested for chemical synthesis. We also explored larger libraries, and ones with other functional modifications, but

these were not readily synthesized and not discussed herein. Autodock tools were used to generate 3D pdbq files with charges and the correct number of rotatable bonds.

The grids for docking was generated using Autodock4 with the grid centered at the position of the platinum atom in the full protein-DNA complex, however, the DNA was removed prior to grid generation. Cubic 22.5Å grids were generated for electrostatics, and vdW parameters for C, S, O, N and polar H with a grid spacing of 0.375Å.

The dockings were performed using Autodock4's defaults for its Lamarckian Genetic Algorithm with a population size of 150, a maximum number of energy evaluations of 5 million and a maximum number of generations of 27000. Each derivative was subjected to 256 runs and ranked according to the best predicted Ki.

c) Results

Rescinnamine as a first lead in inducing MSH2-dependent cell death

We had previously shown that reserpine and rescinnamine are capable of inducing cell death in an MSH2-dependent manner (Vasilyeva 2009, 2010). Further experiments demonstrated that this indole alkaloid is capable of overcoming resistance to cisplatin in ovarian cancer cells (Fig. 1). These initial results suggested rescinnamine to be a good lead toward the development of an effective cancer drug. When applied to a xenograft model, however, the hypotensive activity of the drug prevented administration of statistically effective doses, though a significant trend toward tumor inhibition is observed (Fig. 2). Since none of the commercially available reserpine analogs showed much promise in inducing MSH2-dependent cell death (Vasilyeva et al., 2010), we engaged computational modeling and chemical synthesis to generate novel rescinnamine analogs.

In keeping with our previous studies, we computationally-modeled derivatives of rescinnamine. These computational studies could be used to determine if the activity of rescinnamine could be improved by modification of functional groups on the ring systems, or alternatively not perturbed while perturbing the hypotensive effects of rescinnamine. In this work, the former, changes in activity, is explored. Although there are many possible functionalization sites, and many possible R-groups, in order to avoid a combinatorial explosion computationally, and to restrict our work to experimentally feasible compounds, we restricted ourselves to derivatives that could be readily made as discussed in the methods. The best of these were suggested for chemical synthesis.

Organic Synthesis of rescinnamine analogs

Promising lead molecules that were predicted to induce MSH2-dependent cell death and had a favorable partition coefficient were considered for chemical synthesis (Table 1).

Treatment of methyl reserpate, formed by the basic methanolysis of reserpine (Vasilyeva et al., 2010), with acryloyl chloride yields acryloyl reserpate (**1**) in 35% yield (Figure 3) (Pearce et al., 1989). Similar treatment of **1** with propionoyl chloride yields the saturated derivative **8** (Figure 3). Exposure of **1** to various commercial or synthetic substituted aryl iodides (**9**) to palladium catalyzed Heck coupling conditions gives the di-substituted rescinnamine derivatives (**2-7**) in 27-68% yield and coupling constant analysis shows the exclusive formation of the *trans* stereoisomer (Figure 3) (Ziegler et al., 1978) Condensation of the commercially available methyl esters with hydroxylamine produces the corresponding hydroxamic acids (**10** and **11**, Figure 4) (Hoshino et al., 2009) and Heck coupling of **1** to **10** and **11** in the absence of a

phosphine ligand yields the aniline derivatives of rescinnamine (**9** and **10**, exclusive E stereochemistry) in 35 and 41% yield, respectively rather than the expected hydroxamic acids (Figure 4). (Patel et al., 1977) Such results suggest either a base or palladium-mediated Lossen rearrangement of these hydroxamic acid substrates, a process that holds some literature precedence (Hoshino et al., 2009). Similar Heck coupling of **1** to commercial aryl iodides yields the tri-substituted rescinnamine derivatives **14** and **15** in 70 and 35% yield, respectively (Figure 5). Extensive mass spectrometry and two-dimensional NMR experiments confirm the structure of **14**, which lacks the expected *p*-methoxy group of the starting material (Figure 5). A combination of proton and carbon nuclear magnetic (NMR) spectroscopy, mass spectrometry and elemental analysis confirms the identity of **1-15**.

Effects of rescinnamine analogs on cell viability

We next tested these new rescinnamine analogs in a well-defined cellular system with an endometrial cell line deficient (HEC59) and proficient (via chromosome transfer, HEC59 + chr.2) for MSH2. These assays identified a few compounds that induced cell killing in the micromolar range (Fig. 6, compounds **1**, **6**, **7**, **13**, **15** to a lesser extent **12**). However, little significant MSH2-dependence was observed for these compounds. The only compounds that demonstrated a small difference between MSH2-proficiency and deficiency were **2**, **5** and **13**. No significant difference was detected when determining the IC₅₀ values. These results are surprising in-light of our previous work. Previous studies have suggested that in addition to MSH2-dependent cell-killing due to rescinnamine and derivatives, there is off-target cell-killing as well (Vasilyeva et al., 2010). These results suggest considerable off-target killing for these classes of compounds.

Compound **1**, which lacks the extra ring system, demonstrates activity, but the saturated version of **1** (compound **8**) does not lead to cell killing suggesting that **1** possibly acts through an alternative mechanism (possibly through a Michael reaction) compared to rescinnamine. Taken together, these results demonstrate that the nature of functional groups in key positions of rescinnamine is critical to activity as altering these functional groups can lead to MSH2-independent cell-killing, or even to reduction in IC₅₀ when MSH2 is present, such as with **3** and **12**. These compounds are of particular interest because the difference suggests some sort of interaction or effect due to MSH2, but one that does not lead to cell death.

Another surprising result is the drastic difference between isomers, **3** vs **4** especially, and also to a lesser extent **12** vs **13**. This difference, however, does suggest a preference for electron donating groups at R₂ to avoid a reduction in IC₅₀ when MSH2 is present. In **4** there is an electron donating group (OCH₃) at R₂, and an electron withdrawing group at R₁. In **3**, these groups are switched and there is an ~8-fold change in sensitivity to MSH2. In **13** and **12**, there are electron donating groups at both R₁, and R₂, but when the stronger electron donor is at R₂, there is nearly a 2-fold change in sensitivity to MSH2.

Discussion

We here describe an interdisciplinary approach involving computational simulation, chemical synthesis and cell biology to modify a lead compound, rescinnamine, for the induction of MSH2-dependent cell death in cancer cells.

As evidenced by the results, cell death initiation itself, and more so dependence on MSH2, is largely dependent on specific functional groups, rather than just the presence of ring systems. Small changes to the structure of rescinnamine affect its activity on cell death significantly. This

represents a great challenge to further work in developing derivatives as rescinamine, but does suggest three possible avenues for further research. First, a search for the off-targets that are being affected so as to design away off-target interactions. Second, a study as to why MSH2 sensitizes cells to two compounds (**3** and **12**). These may be possible candidates for further study, if they interact with MSH2, but not in a manner to promote cell death. Third, exploration of other functional sites away from the phenyl ring for modification.

d) Acknowledgements

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f) Figures

Fig. 1. Effect of rescinnamine on viability of cisplatin-resistant cells. **A:** Structure of rescinnamine. **B:** The ovarian cancer cell line A2780 and its cisplatin-resistant derivative

were exposed to increasing concentrations of cisplatin (left) and rescinnamine (right). Cell survival was determined in relation to untreated cells.

Fig. 2. Effect of rescinnamine on tumor growth in a mouse xenograft model. A: Images of mice untreated and treated with several different concentrations of rescinnamine. Tumors were obtained by injection with the colorectal cancer cell line SW620. **B, C:** Graphs illustrating tumor growth and body weight of untreated and treated mice in two different cell systems (colorectal cancer (B), endometrial cancer (C)).

Fig. 3. Synthetic procedures for the preparation of rescinnamine derivatives (1-8). Condensation reactions between methyl reserpate and acyl chlorides to give **1** and **8**. Heck coupling procedure with **1** to yield rescinnamine derivatives **2-7**.

Fig.4. Synthetic procedures for the preparation of rescinnamine derivatives (12-13). Heck coupling of synthetic hydroxamic acid aryl iodides gives the amino-substituted rescinnamine derivatives **12** and **13**.

Fig. 5. Synthetic procedures for the preparation of rescinnamine derivatives (14-15). Heck coupling of commercially available aryl iodides to give rescinnamine derivatives **14** and **15**.

Fig. 6. Cell Survival of MSH2 proficient and deficient cells after treatment with rescinnamine analogs. HEC59 and its isogenic cell line containing a chromosome 2 transfer were treated with increasing concentrations of the indicated compound (Table 1). Cell viability is graphed in dependence of concentration.

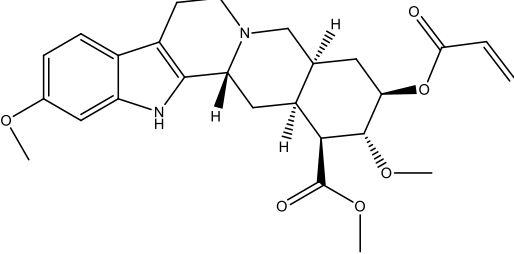
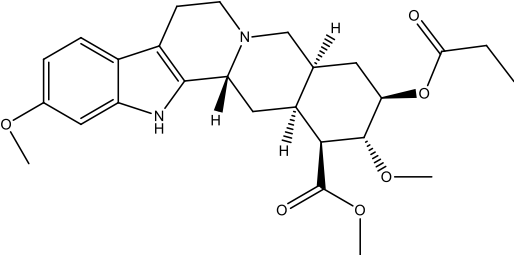
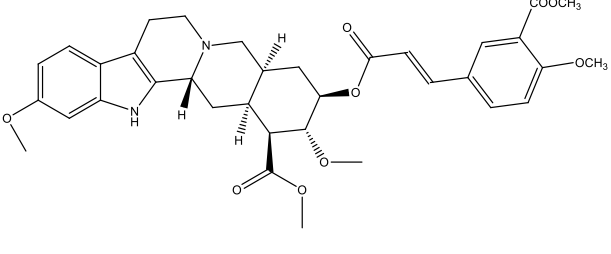
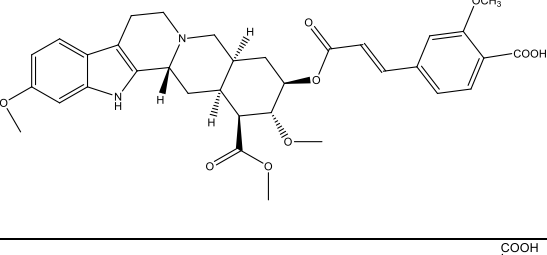
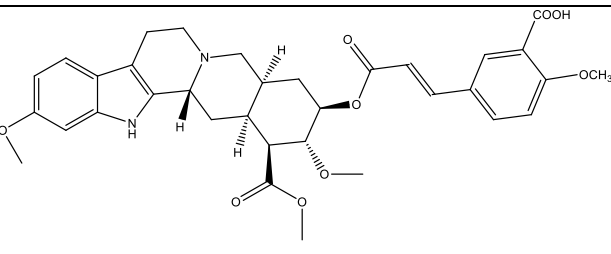
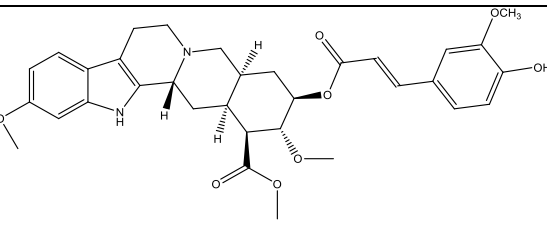
Fig 7. Structure of Rescinnamne with sites for computational modification.

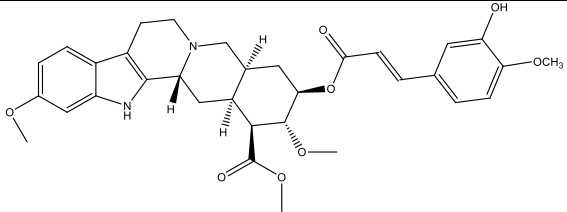
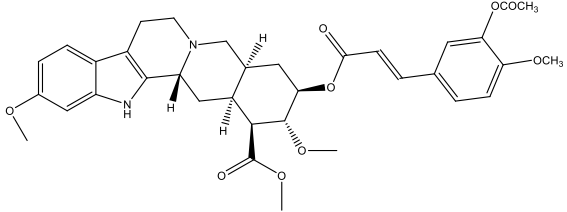
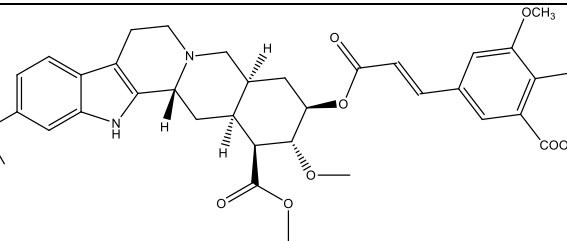
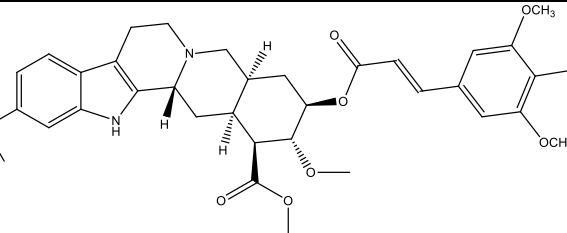
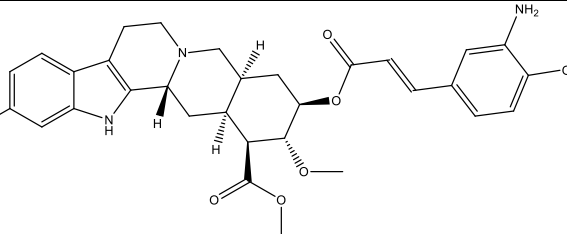
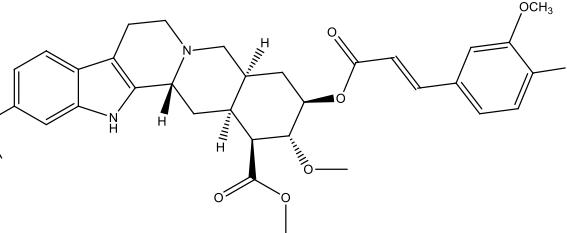
g) Tables

Table 1. Rescinnamine Analogs.

IC50 values for individual compounds. Where appropriate, graphs that did not reach 50% mortality were extrapolated. NA: no extrapolation possible.

			IC50	
Entry	Compound Number	Compound	MSH2 deficient	MSH2 proficient

	1		20.7	20.37
	8		NA	NA
	2		50.72	48.91
	3		163.1	1223
	4		89.29	84.23
	5		NA	NA

6		22.32	21.29
7		39.28	NA
14		NA	NA
15		47.67	54.53
12		77.25	113.4
13		85.73	68.38

NA: not applicable, since data did not reach below 50%.

