Em08red, a dual functional antiproliferative emodin analogue, is a downregulator of ErbB2 expression and inducer of intracellular oxidative stress

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Abstract: Expression of ErbB2 protein is inversely correlated with the prognosis in cancer patients. Consequently, strategies targeting ErbB2 remain an attractive option in treating several types of malignancies, including oral cancer. In addition, many studies have shown that emodin and emodin derivatives are able to inhibit growth of ErbB2-overexpressing tumor cells. In this study, a series of computer modeling-generated emodin analogues were synthesized and tested for their antiproliferative activity against oral cancer cell lines overexpressing ErbB2. Among these analogues, em08red (1,8-dihydroxy-9(10H)-anthracenone) demonstrated potent antiproliferative activity against all three tested ErbB2-overexpressing cell lines, ie, FaDu, HSC3, and OECM1. Treatment with em08red significantly downregulated activation of ErbB2 as well as the ErbB2 protein expression level in the tested cell lines and induced G2 arrest. Antiapoptosis protein (Bcl-xl and Bcl-2) expression levels were also downregulated, and active caspase-3 and caspase-9 was detected in cells after treatment with em08red. Moreover, treatment with em08red stimulated production of cytotoxic reactive oxygen species in treated cells, and this could be partially reversed by pretreatment with N-acetylcysteine. Overall, we demonstrated inhibition of ErbB2 function and induction of reactive oxygen species in tumor cells by em08red, which prevented proliferation of tumor cells and induced apoptotic cell death.

Keywords: ErbB2, emodin, em08red, reactive oxygen species, structure-activity relationship

Introduction

Oral cancer, the sixth most common cancer worldwide, is becoming a serious issue in global cancer epidemiology, especially in South and Southeast Asia and in Pacific regions.1 Most prevalent malignancies affecting the oral cavity are squamous cell carcinomas, and the incidence of oral cancer is higher in men than in women in many countries.2 Heavy smoking, alcohol abuse, and chewing of betel nut contribute to the high occurrence of oral cancer in most countries. Among the risk factors, chewing of betel nut is a widespread habit among adult men aged 25–55 years in Taiwan, and is the major element contributing to the oral cancer incidence in men of 29.2 per 100,000.3,4 In clinic, surgical removal or radiotherapy alone is frequently used to treat early (stage I and II) oral cancer, but surgery combined with radiotherapy and follow-up chemotherapy are often required for advanced (stage III and IV) oral cancers.5 However, the overall treatment of oral cancers is unsatisfactory and the 5-year survival rate for advanced oral cancers under multimodal therapy is as low as ~12%.6 The high incidence and mortality associated with oral cancers has a heavy social and economic impact, and these cancers have become a major health issue for the government and health care agencies in Taiwan. Further, due to the high prevalence of oral cancers...
that become chemoresistant and radioresistant, new effective antitumor agents are urgently required.

Aggregation of genomic alterations leads to aberrant activation of oncogenes and/or inactivation of tumor suppressors, contributing to the development of cancer. Recently, ErbB2 gene amplification and elevated ErbB2 function was observed in clinical oral cancer specimens or patient serum,9–11 and increased ErbB2 expression is strongly correlated with the progression of cancer and a poor prognosis.12 The phosphorylation level of ErbB2 also serves as a poor prognostic indicator of survival in patients with cancer and is associated with resistance to gefitinib in cancer cells.13,14 Therefore, suppression of ErbB2 signaling has become an attractive therapeutic target when treating cancer patients with overactivated ErbB2, and many ErbB2-targeted drugs have been used in the clinic or are under active development.

Emodin, a well known anthraquinone and abundant in many foods and medicinal herbs, has been reported to have a variety of biological functions, including laxative, antimicrobial, redox regulatory, and antitumor properties.15 Several studies have shown that emodin enhances the therapeutic effects of several chemotherapeutic agents, including cisplatin, doxorubicin, etoposide, and paclitaxel, in ErbB2-overexpressing cancer cells.16 Moreover, an emodin azide methyl anthraquinone derivative has been shown to disturb the binding between ErbB2 and heat shock protein 90, resulting in proteasomal degradation of ErbB2;17 however, the detailed molecular mechanisms are still unknown. With computer modeling, a series of emodin analogues capable of occupying the ErbB2-ATP binding pocket were synthesized and evaluated for their antiproliferative efficacy against several oral cancer cell lines. A small molecule, em08red (1,8-dihydroxy-9(10H)-anthracenone), showed a low IC_{50} value (<10 µM) in three ErbB2-overexpressing oral cancer cell lines and dramatically decreased ErbB2 protein levels. Treatment with Em08red impeded cell cycle progression at G2 phase, with downregulated expression of Cdk1 and cyclin B. Moreover, signatures of apoptosis, including the sub-G1 population, phosphatidylserine exposure, caspase activation, and loss of antiapoptosis molecules (Bcl-xl and Bcl-2), were also observed after treatment with em08red. Further experiments also demonstrated that treatment with em08red induced reactive oxygen species (ROS), resulting in cell death. Overall, em08red, an emodin analogue, represents a novel ErbB2-targeting drug lead. Further more potent compounds of the em08red series for ErbB2-overexpressing oral cancers are under active investigation.

Materials and methods
Cell culture
Human tongue carcinoma (HSC3), human hypopharyngeal carcinoma (FaDu), and human oral squamous carcinoma (OECM1) cell lines were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). HSC3 and FaDu cells were maintained in minimum essential medium containing 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate, and OECM1 cells were cultured in Roswell Park Memorial Institute-1640 medium with the same supplements as mentioned above. All cells were cultured in a 5% CO_{2} humidified incubator set at 37°C.

Antibodies and chemicals
Antibodies against Bcl-2, Bak, Bcl-xl, and phosphor-MPM2 were obtained from Millipore (Billerica, MA, USA). Caspase antibodies and antibodies against phosphor-ErbB2-Y1221/1222, phosphor-ErbB2-Y1248, and total ErbB2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Actin antibody was acquired from Santa Cruz (Dallas, TX, USA). All chemicals used were of molecular or analytical grade and were purchased from Sigma-Aldrich (St Louis, MO, USA) or USB Corporation (Santa Clara, CA, USA).

General procedure for preparation of emodin analogues
Anhydrous AlCl₃ and prebaked NaCl were mixed in a 2:1 molar ratio and heated to 110°C until molten. After phthalic anhydride/substituted phenol mixtures were gradually added to the AlCl₃/NaCl melt, the reaction temperature was gradually increased to and maintained at 160°C for 5 hours and then cooled to 0°C in an ice bath. Next, 20 mL of 10% HCl was added, stirred at 0°C for 15 minutes, and then refluxed at 100°C for 30 minutes. The reaction mixture was cooled to room temperature and extracted with ethyl acetate. The final product was purified by silica gel column chromatography (EtOAc/n-Hex =1:1).

Determination of antiproliferative efficacy
A modified MTT (3-(4,5-carboxymethoxy-2-y)-2,5-diphenyl tetrazolium bromide) method was used to determine the efficacy of antitumor activity, and the IC_{50} value was calculated.18 Briefly, tumor cells (3,000 cells/well) were seeded into 96-well plates and then treated with vehicle or various concentrations of the test compounds for 72 hours. Two hours...
before the end of incubation, MTT at a final concentration of 5 µg/mL was added. Afterwards, solubilization buffer (40% dimethylformamide and 20% sodium dodecyl sulfate in H2O) was added to the wells to dissolve the violet formazan precipitation overnight at 37°C. Absorbance at 570 nm was then detected by a microplate reader (Molecular Devices, Sunnyvale, CA, USA) and the IC50 value was calculated by linear regression analysis.

Cell cycle analysis
Em08red-treated and control cells were trypsinized, collected, and fixed with 70% ice-cold ethanol in phosphate-buffered saline. After overnight incubation at −20°C, the cells were collected, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 15 minutes at room temperature, and then stained with propidium iodide 40 µg/mL and RNase A (0.1 mg/mL) in phosphate-buffered saline for 30 minutes at 37°C. After the free propidium iodide was removed by centrifugation, the samples were analyzed immediately on a Canto II flow cytometer (BD Biosciences, San Jose, CA, USA) and the proportion of each cell cycle phase was analyzed by FCS express software (De Novo, Los Angeles, CA, USA).

Apoptotic cell death detection
An annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Enzo, Farmingdale, NY, USA) was used to monitor apoptotic cells according to the manufacturer’s manual. Briefly, samples were collected and resuspended in staining buffer containing 1 mg/mL propidium iodide and 0.025 mg/mL annexin V-FITC at room temperature for 15 minutes in the dark. The samples were then immediately analyzed on a Canto II flow cytometer and the proportion of each population was calculated using FCS express software. The annexin V-positive and annexin V/propidium iodide double-positive populations were considered as apoptotic cells.

Caspase activity measurement
Activation of caspase-3, caspase-8, and caspase-9 in living cells was evaluated by the CaspGLOW™ fluorescein active caspase staining kit (BioVision, Milpitas, CA, USA) according to the manufacturer’s protocols. Briefly, treated cells were incubated with medium containing caspase fluorescent substrates for 2 hours at 37°C. After trypsinization and centrifugation, samples were resuspended in wash buffer and analyzed on a Canto II cytometer. The acquired data were then analyzed using the FCS express software.

Intracellular ROS level measurement
To determine the amount of intracellular H2O2, the treated cells were incubated with 20 µM of 2',7'-dichlorodihydrofluorescein diacetate for 30 minutes at 37°C prior to analysis. Cells were then trypsinized, resuspended in phosphate-buffered saline, and analyzed on a Canto II cytometer. The acquired data were then analyzed by the FCS express software.

Western blot analysis
Cells were collected and lysed with cell lysis buffer (150 mM NaCl, 20 mM Tris-Cl pH 8.0, 0.5% NP-40, 1 mM phenylmethanesulfonyl fluoride, 1 mM NaF, 1 mM Na3VO4, and 20 µg/mL aprotinin in distilled H2O) and the total protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Scientific, Swedesboro, NJ, USA). Equal amounts of total protein (30–40 µg) were resolved in 10%–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and then transferred to polyvinylidene difluoride membranes. After blocking with 5% bovine serum albumin/ TBS-Tween for one hour at room temperature, the membrane was incubated with primary antibodies overnight at 4°C. Subsequently, horseradish peroxidase-conjugated secondary antibodies were reacted with the membrane for one hour at room temperature. After washing, signals were acquired on a LAS-4000 biomolecular imager (Fujifilm, Tokyo, Japan).

Statistical analysis
All experiments were carried out at least three times independently and the results are presented as the mean ± standard deviation. The Student’s t-test was used to determine the significance of the differences. A P-value less than 0.05 was considered to be statistically significant.

Results
Synthesis of emodin analogues
Anthraquinones 1–9 was synthesized by reaction of selected phthalic anhydrides with substituted phenols in the presence of AlCl3/NaCl (Figure 1A). Compound 7 and emodin were then treated with SnCl2 in the presence of HCl to generate compounds 10 and 11, respectively (Figure 1B).

Antiproliferative activity of emodin analogues
To identify emodin analogues with potent antiproliferative activity against oral cancers, eleven compounds were synthesized and purified, and their structures were determined. The IC50 value for each compound was determined in three
ErbB2-overexpressing oral cancer cell lines (FaDu, HSC3, and OECM1 cells) using the MTT assay. Compounds with an IC\textsubscript{50} value over 50 \(\mu\)M were considered not to be cytotoxic towards oral cancer cells. As shown in Table 1, compounds with no hydroxyl substitution (compound 1) or with three hydroxyl substitutions (compound 9) displayed very weak antiproliferative activity. Compounds 5 and 7 had an IC\textsubscript{50} value of \(\sim 15 \mu\)M in FaDu cells, but both compounds showed very weak cytotoxicity towards the other two tested cell lines. However, compounds with two hydroxyl substitutions (compounds 4, 6, and 8) showed very little antiproliferative activity against oral cancers. Compound 7 demonstrated the most potent cytotoxicity of all the compounds synthesized and was chosen for further reduction to generate compound 10 (em08red). The antiproliferative assay results for em08red showed potent antiproliferative activity towards all three oral cancer cell lines, with an IC\textsubscript{50} value below 10 \(\mu\)M. Therefore, this compound was chosen for further study.

**Treatment with em08red decreased ErbB2 protein levels**

Overactivation of the ErbB2 signaling pathway has a critical role in the development of oral cancer and survival. Previous reports indicate that emodin may serve as an adjuvant treatment for ErbB2-overexpressing cancer cells.\textsuperscript{9,19} Therefore, all compounds with antiproliferative activity were tested for their
influence on ErbB2 activity. Phosphor-ErbB2 protein levels and total-ErbB2 expression were determined by Western blot analysis (data not shown). Among the analogues synthesized, only em08red showed significant inhibition of ErbB2 activity. As shown in Figure 2A and B, after 36 hours of treatment, ErbB2 expression levels were dramatically reduced and the level of ErbB2 phosphorylation at tyrosine 1221/1222 and 1248, which both account for activation of ErbB2 receptors,

was below the level of detection in FaDu and HSC3 cells. The downregulatory effect of em08red on ErbB2 protein was also observed in the treated OECM1 cells (data not shown). Our results demonstrated effective inhibition of ErbB2 activity by em08red, warranting further investigation.

**Em08red induced G2/M phase arrest and intrinsic apoptosis**

A previous study showed that downregulation of ErbB2 protein expression by treatment with neu differential factor could induce arrest of tumor cells in G2/M phase in cells overexpressing ErbB2.21 To determine if em08red has a similar pharmacological effect on cell cycle progression, em08red-treated cells were subjected to flow cytometry analysis. The data indicated significant G2/M accumulation after 24 hours of treatment with em08red in both FaDu and HSC3 cell lines (Figure 3A). G2/M phase-associated proteins, Cdk1 and cyclin B,22 were downregulated after treatment with em08red and phosphor-MPM2 signals were also reduced after em08red, indicating that the treated cells were blocked at G2 phase (Figure 3B). Additionally, we observed a population of sub-G1 em08red-treated HSC3 and FaDu cells after 24 and 48 hours of treatment, respectively, indicative of G2 phase arrest and cell death (Figure 3A). To determine if the molecular mechanisms of em08red-induced cell death occurred via apoptosis or necrosis, the phosphatidylserine level (a marker of apoptosis) on the outer membrane of em08red-treated cells was examined by annexin V staining. As shown in Figure 4A, the percentage annexin V-positive population in em08red-treated FaDu and HSC3 cells increased in a time-dependent manner and correlated with our observations on sub-G1 cell cycle analysis.

Activation of caspase-8 and caspase-9 is a hallmark of extrinsic and intrinsic apoptotic cascade pathways, respectively.23 To identify which apoptotic signaling is involved, caspase activation in em08red-treated FaDu cells was monitored using caspase-specific fluorogenic substrates and Western blots. As shown in Figure 4B, both caspase-3 and caspase-9 were activated, whereas no activation of caspase-8 was detected. Matching Western blot analysis results also demonstrated that em08red could induce cleavage and activation of caspase-3 and caspase-9 but not caspase-8 (Figure 4C). Moreover, Bcl-2 and Bcl-x, which are antiapoptosis molecules involved in the intrinsic apoptosis pathway, were decreased in the oral cancer cell line after treatment with em08red (Figure 4D). Collectively, our observations indicate that treatment with em08red induced cell cycle arrest at G2

<table>
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<td>2</td>
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<td>&gt;50</td>
<td>&gt;50</td>
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<tr>
<td>9</td>
<td>40.9±18.1</td>
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<tr>
<td>10 (em08red)</td>
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<td>9.8±5.7</td>
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<td>46.0±0.3</td>
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**Notes:** Treated FaDu (A) and HSC3 (B) cells were harvested at the indicated times and total cell lysates were subjected to Western blot analysis. Antibodies specifically against total ErbB2 and phosphorylation sites of ErbB2 at tyrosine 1221/1222 or tyrosine 1248 were applied to detect protein expression after treatment. Actin expression served as the loading control.

**Figure 2** Treatment with em08red downregulates phosphorylation and expression of ErbB2.
phase and subsequently triggered the intrinsic apoptosis pathway in ErbB2-overexpressing oral cancer cells.

Em08red caused cell death via production of ROS

Many anthraquinones have been reported to cause ROS-mediated apoptosis,24,25 and it has also been shown that perturbation of ErbB2 activity in non-small-cell lung cancers can contribute to elevation of ROS and cell death.26 Hence, whether ROS production is also involved in em08red-mediated cytotoxicity was investigated to clarify the mechanism of action further. As shown in Figure 5A, treatment with em08red significantly increased the intracellular ROS level. To determine whether em08red-mediated cytotoxicity is ROS-dependent, cells were pretreated with N-acetylcysteine, a free radical scavenging agent, for 2 hours followed by treatment with em08red for 24 hours. Our results showed that pretreatment with N-acetylcysteine could partially inhibit the cytotoxic effects of em08red (Figure 5B), suggesting that production of ROS accounts for a portion of em08red-induced cell apoptosis.

Em08red induced proteasomal degradation of ErbB2 protein

To clarify further whether em08red-induced ErbB2 downregulation may also occur via proteasomal regulation, MG132, a proteasome inhibitor, was used to hinder proteasome activity in the presence of em08red. As shown in Figure 6, treatment with MG132 could restore ErbB2 expression in em08red-treated cells, indicating that em08red-induced ErbB2 downregulation could occur in a proteasome-dependent manner.

Discussion

Extensive investigation and progressive clinical application of target-specific small molecule inhibitors of kinase activity has now been well documented in many malignancies. Gefitinib and erlotinib are two effective pioneer drugs with activity...
Figure 4 Treatment with em08red triggered the intrinsic apoptotic pathway and suppressed the antiapoptosis molecules.

Notes: (A) Cells were treated for 24, 48, and 72 hours and then harvested for detection of the annexin V-positive population by flow cytometry. (B) FaDu cells were treated with em08red (10 µM) for 24 hours, and intracellular caspase-3, caspase-8, and caspase-9 activity was detected using a CaspGLOW™ fluorescein active caspase staining kit. Control cells were incubated with vehicle only (0.1% dimethyl sulfoxide). The data are presented as the fold increase in treated samples and the asterisk indicates a significant difference between the treated group and the control group (P<0.05). (C) Treated FaDu cells were harvested and lysed at the indicated times. Equal amounts of total cell lysates were subjected to Western blot analysis. Antibodies specific for cleaved caspase-3, caspase-8, or caspase-9 were used in the experiment. Actin expression served as the loading control. (D) Total lysates extracted from treated FaDu cells were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After transferring to the membranes, antibodies specific for Bcl-2, Bcl-x, Bak, and actin were reacted with the indicated membranes. Actin expression served as the loading control. All Western blotting images were acquired on an LAS-4000 biomolecular imager.
against cancers overexpressing epidermal growth factor receptor, a member of the ErbB family.\textsuperscript{27} Overactivation of ErbB2 is believed to participate in the development and progression of oral cancers and many other types of malignancy, so is an attractive therapeutic target.\textsuperscript{10} Kinase inhibitors targeting ErbB2 or the epidermal growth factor receptor/ErbB2 signaling pathway have become a top research priority in the treatment of cancer.\textsuperscript{28}

Previously, emodin has been reported to induce apoptosis or growth inhibition in a number of cancer cell lines,\textsuperscript{29,30} including some ErbB2-overexpressing breast cancer cells. Treatment with emodin sensitizes cells to treatment with paclitaxel,\textsuperscript{19} but the details of the molecular mechanism involved remain elusive. Recently, studies have also shown that an azide methyl containing emodin could promote proteasomal degradation of ErbB2 and induce cell death.\textsuperscript{17} Consequently, emodin is an attractive pharmaceutical reagent for use to disrupt ErbB2 activity in oral cancers. In this study, a computer-aided design approach was used and molecules that best fitted the ATP-binding pocket of ErbB2 were synthesized and evaluated for antiproliferative activity in ErbB2-overexpressing oral cancer cell lines. Initially, the antiproliferative effects of analogues with hydroxyl substitutions at different sites on the anthraquinone were examined (Figure 1 and Table 1). A preliminary structure-activity relationship study suggested compounds with no hydroxyl substitution (compound 1) or more than three hydroxyl substitutions (compound 9, emodin) displayed very weak antiproliferative activity. The compounds containing one or two hydroxyl groups showed increased antiproliferative activity; however, the results indicated that hydroxyl substitution at the R1 position would be necessary for antiproliferative activity (compounds 2, 5, 6, and 7) but hydroxyl substitution at position 2 seemed to reduce the potency of the analogues (compounds 3, 4, 8 and 9). Additional hydroxyl substitutions at position 4 or 8 augmented the potency of compounds (5 and 7). As shown in Figure 1 and Table 2, it is speculated that the number of hydroxyl substitutions might govern the potency of emodin analogues with regard to anticancer activity in oral malignancies ($2>0>3$). Conversely, simply reduction of ketone at position 10 of emodin (compound 11) did not improve the potency of antiproliferative activity, but reduction of ketone at position 10 of the two hydroxyl-substituted analogue (compound 7) for compound 10, designated as em08red, showed greatly enhanced antiproliferative activity towards all three oral cancer cell lines, with an IC$_{50}$ value below 10 µM. Em08red was therefore chosen for further study.
Our results clearly show that treatment with em08red significantly blocks activation of ErbB2 by reducing ErbB2 protein levels via proteasomal degradation (Figures 2 and 6), implying a shared characteristic of some anthraquinones in affecting ErbB2 function or stability and contributing to retardation of G2. Our observation was similar to previous findings of downregulation of ErbB2 and G2/M arrest via neu differential factor in an ErbB2-overexpressing breast cancer cell line.21 Other studies have shown that generation of ROS is an essential product for anthraquinone-mediated cell death in several cancer cell lines.22-24,31 Further investigation showed that treatment with em08red increased intracellular ROS levels and that induction of ROS had a causative role in em08red-induced cell death (Figure 5). In addition, our results demonstrate that em08 red-mediated apoptosis reduces ErbB2 protein expression and is accompanied by induction of ROS (Figures 2 and 5A). Both mechanisms have previously been shown to induce apoptosis.30,31 To the best of our knowledge, em08red is the first anthraquinone that can simultaneously downregulate expression of ErbB2 protein and efficiently induce production of ROS (Figure 2). Whether the molecular mechanisms of ErbB2 downregulation by treatment with em08red occurs via reactive oxygen species (ROS)-involved lysosomal degradation or enhanced ubiquitination process of ErbB2 is still under active investigation.

A fascinating and efficient pharmacore will be established and further development of potential analogues has been launched for further study. In conclusion, our data provide a novel scheme of emodin analogue effectively targeted ErbB2 protein and imply a developmental future of anthraquinones on target therapy.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

References


Supplementary materials
Chemistry

1,4-dihydroxyanthraquinone (compound 1)
Yield 82%. Melting point 194°C–195°C. MS: 240 (M+). FTIR: 3,065, 1,630, 1,590 cm⁻¹. 1H NMR (DMSO-d6, 400 MHz): δ 12.67 (s, 1H), 10.86 (s, 1H), 8.11–8.18 (m, 2H), 7.88–7.89 (m, 2H), 7.47 (s, 2H). Anal. Calcd for C₁₄H₁₀O₄: C, 70.00; H, 3.36. Found: C, 69.98; H, 3.35.

1,8-dihydroxyanthraquinone (compound 2)
Yield 77%. Melting point 194°C–187°C (decomposes). MS: 240 (M+). FTIR: 1,676, 1,668, 1,527 cm⁻¹. 1H NMR (DMSO-d6, 400 MHz): δ 11.88 (s, 2H), 7.75 (d, 2H, J=7.6 Hz), 7.68 (t, 2H, J=7.6 Hz), 7.36 (d, 2H, J=7.6 Hz). Anal. Calcd for C₁₄H₁₀O₄: C, 70.00; H, 3.36. Found: C, 70.01; H, 3.37.

1,5-dihydroxyanthraquinone (compound 3)
Yield 82%. Melting point 94°C–96°C. MS: 240 (M+); FTIR: 3,322, 1,680 cm⁻¹. 1H NMR (DMSO-d6, 400 MHz): δ 10.97 (s, 2H), 8.05 (d, 2H, J=8.5 Hz), 7.48 (d, 2H, J=2.4 Hz), 7.19 (dd, 2H, J=2.4, 8.5 Hz). Anal. Calcd for C₁₄H₁₀O₄: C, 70.00; H, 3.36. Found: C, 70.02; H, 3.37.

2,6-dihydroxyanthraquinone (compound 4)
Yield 82%. Melting point 348°C–349°C. MS: 240 (M+). FTIR: 3,322, 1,597 cm⁻¹. 1H NMR (DMSO-d6, 400 MHz): δ 10.97 (s, 2H), 8.24 (dd, 1H, J=1.17, 8.26 Hz), 8.17 (dd, 1H, J=1.50, 7.63 Hz), 8.12–8.16 (m, 2H), 7.92–7.97 (m, 2H), 7.81 (t, 1H, J=7.60, 7.60 Hz), 7.71 (d, 1H, J=7.60 Hz), 7.38 (d, 1H, J=7.60 Hz). Anal. Calcd for C₁₄H₁₀O₄: C, 75.00; H, 3.60. Found: C, 75.02; H, 3.61.

2-hydroxyanthraquinone (compound 8)
Yield 79%. Melting point 195°C–196°C. MS: 224 (M+). FTIR: 3,370, 1,680 cm⁻¹. 1H NMR (DMSO, 400 MHz): δ 8.49 (s, 1H), 8.08–8.11 (m, 2H), 8.03 (d, 1H, J=8.56 Hz), 7.78–7.88 (m, 2H), 7.43 (d, 1H, J=2.40 Hz), 7.18 (dd, 1H, J=2.40, 8.56 Hz). Anal. Calcd for C₁₄H₁₀O₄: C, 70.00; H, 3.60. Found: C, 75.01; H, 3.62.

Anthrquinone (compound 9)
Yield 85%. Melting point 284°C–286°C. MS: 208 (M+). FTIR: 1,686, 1,592 cm⁻¹. 1H NMR (DMSO, 400 MHz): δ 12.40 (s, 1H), 12.18 (s, 1H), 10.79 (s, 1H), 10.63 (s, 1H), 8.56, 8.56 Hz), 8.12–8.16 (m, 2H), 7.92–7.97 (m, 2H), 7.70 (t, 1H, J=7.60 Hz), 7.60 (d, 1H, J=7.60 Hz). Anal. Calcd for C₁₄H₁₀O₄: C, 75.00; H, 3.60. Found: C, 75.02; H, 3.61.

1,8-dihydroxy-10H-anthracene-9-one (compound 10, emd08red)
SnCl₂ solution (13.19 mmol in 37% HCl) was added dropwise over a 3-hour period into refluxed 1,8-dihydroxyanthracene-9,10-dione (0.7 mmole in glacial acetic acid). The solution was then cooled, and the resulting crystals were collected by filtration. Purification by column chromatography (EtOAc/n-Hex =1:1) provided the yellow platelets: 71% yield; melting point 144°; MS: 226 (M+); FTIR: 1,616, 1,597 cm⁻¹; 1H NMR (CDCl₃): δ 12.26 (1H, s, 1,8-OH), 7.47 (2H, dd, J=15.6, 15.6, H-3,6), 6.88 (4H, d, J=15.6, H-2,4,5,7), 4.33 (2H, s, H-10); Anal. Calcd for C₁₄H₁₀O₃: C, 74.33; H, 4.46. Found: C, 74.37; H, 4.45.

1,3,8-trihydroxy-6-methyl-10H-anthracene-9-one (compound 11)
Emodin (1.35 g, 5 mmol) was reacted with SnCl₂ (5.0 g, 26 mmol) as described for the preparation of 11 to afford 12 (1.02 g, 80%) in the form of yellow plates. MS: 256 (M+); FTIR: 1,611, 1,590 cm⁻¹. Melting point 250°C (decomposes). 1H-NMR (DMSO-d6, 400 MHz) δ 12.33 (s, 1H), 12.18 (s, 1H), 10.79 (s, 1H), 6.74 (s, 1H), 6.64 (s, 1H), 6.38 (s, 1H), 6.18 (s, 1H), 4.27 (s, 2H), 2.28 (s, 3H). Anal. Calcd for C₁₃H₁₂O₄: C, 70.31; H, 4.72. Found: C, 70.33; H, 4.73.