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Soluble guanylate cyclase stimulators increase sensitivity to cisplatin in head and neck squamous cell carcinoma cells

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is an aggressive and often fatal disease. Cisplatin is the most common chemotherapeutic drug in the treatment of HNSCC, but intrinsic and acquired resistance are frequent, and severe side effects occur at high doses. The second messenger cyclic GMP (cGMP) is produced by soluble guanylate cyclase (sGC). We previously reported that activation of the cGMP signaling cascade caused apoptosis in HNSCC cells, while others found that this pathway enhances cisplatin efficacy in some cell types. Here we found that sGC stimulators reduced HNSCC cell viability synergistically with cisplatin, and enhanced apoptosis by cisplatin. Moreover, the sGC stimulators effectively reduced viability in cells with acquired cisplatin resistance, and were synergistic with cisplatin. The sGC stimulator BAY 41-2272 reduced expression of the survival proteins EGFR and β -catenin, and increased pro-apoptotic Bax, suggesting a potential mechanism for the anti-tumorigenic effects of these drugs. The sGC stimulator Riociguat is FDA-approved to treat pulmonary hypertension, and others are being studied for therapeutic use in several diseases. These drugs could provide valuable addition or alternative to cisplatin in the treatment of HNSCC.

Keywords

cGMP; sGC; HNSCC; YC-1; BAY 41- 2272; apoptosis; EGFR; β -catenin

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Conflict of Interest

The authors have nothing to declare.

1. Introduction

Head and neck cancer (HNC), the sixth most common cancer, are diverse tumors within the oral cavity, oropharynx, hypopharynx and larynx [1-3]. Over 500,000 patients are diagnosed annually worldwide, with squamous cell carcinoma (HNSCC) representing 90% of the cases. Long term survival varies from 10-50%, depending on tumor size, stage and resectability, and is less than 10 months for recurrent diseases. Excessive alcohol and tobacco use are major risk factors, with human papillomavirus (HPV) infection accounting for ~25% of the cases [3, 4]. Surgery and radiotherapy are the mainstay treatments for small tumors, while combined radiotherapy and chemotherapy are employed in advanced disease. Many tumors overexpress epidermal growth factor receptor (EGFR), and are treated with various EGFR antagonists [5, 6]. In spite of recent advances, prognosis for recurrent/metastatic HNSCC remain dismal. Furthermore, disease progression or treatment can severely reduce the quality of life by causing facial disfigurement and impeding eating and speech [7].

The efficacy of chemotherapy is often compromised by intrinsic or acquired resistance [8]. Indeed, resistance to cisplatin, the most common chemotherapeutic drug in the treatment of HNC, necessitates the use of increased therapeutic doses, resulting in escalation of adverse side effects. There is, therefore, an urgent need for novel therapeutics in the treatment of HNSCC, which also can overcome resistance to cisplatin.

The cyclic GMP (cGMP)/protein Kinase G (PKG) pathway serves as an endogenous apoptotic pathway in breast, colon and glioma [9-14]. Cyclic GMP is generated from GTP by two distinct guanylate cyclases (GC): particulate, which are membrane receptors for natriuretic peptides [15], and soluble (sGC), which are cytosolic receptors for nitric oxide (NO) [16]. Once produced, cGMP can be rapidly hydrolyzed by cyclic nucleotide phosphodiesterases (PDEs), such as PDE5 [17]. The main downstream effectors of cGMP are two serine-threonine kinases, PKG-I and PKG-II [18]. Several drugs which target the cGMP/PKG pathway are approved for the treatment of nonmalignant conditions. For example, Riociguat, a NO-independent sGC stimulator, is used to treat pulmonary hypertension [19], while selective PDE5 inhibitors such as Tadalafil (Cialis) and Sildenafil (Viagra) are used to treat erectile dysfunction [20]. Thomson *et al* [21] were the first to link the cGMP/PKG pathway to inhibition of growth of colon cancer cells by a mechanism involving the suppression of β -catenin. We recently reported that activation of the cGMP pathway in HNSCC cells by sGC activators or PDE5 inhibitors reduced cell viability and clonogenic growth and induced apoptosis [22]. Others found that YC-1, a sGC activator, as well as PDE5 inhibitors, enhance the efficacy of a variety of chemotherapeutic agents [23-25]. In addition, activation of the cGMP/PKG pathway reduces signaling via the EGFR [26] and β -catenin [27], pathways, both of which have been associated with HNSCC cell survival and chemoresistance [28, 29].

The objectives of this study were to: 1) determine whether sGC activators enhance the efficacy of cisplatin to reduce cell viability and increase apoptosis in HNSCC cells, 2) examine the effects of sGC activators on cisplatin resistant cells, and 3) explore the effects of

these drugs on signaling and pathways such as EGFR and β -catenin, and on apoptotic mediators such as Bax and Bcl-2.

2. Materials and Methods

2.1 Cell lines and culture

UMSCC-1 (UM1; oral cavity, HPV-) and UMSCC-47 (UM47; tongue, HPV+) cells were purchased from the University of Michigan. CAL27 (tongue, HPV-) parental cells and their cisplatin-resistant line (CAL27-CisR) were from Pawan Kumar at Ohio State University. Cells were maintained in DMEM containing 10% FBS (Atlanta Biologicals, Flowery Branch, GA) and 50 μ g/ml normocin (Invivogen, San Diego, CA). UM1 cells were cultured in increasing concentrations of cisplatin for 6 months to produce a cisplatin resistant subline UM1-CisR. CAL27-CisR and UM1-CisR cells were maintained in media containing 3 μ M cisplatin. For experiments, cells were starved for 24 h and then incubated with various treatments in DMEM containing 2% charcoal/dextran-stripped FBS (Atlanta Biologicals) unless otherwise noted.

2.2 Cell viability

Cells were plated at 3000 cells/well in 96-well plates and treated for 72 h with the sGC stimulators BAY 41-2272 (BAY), YC-1 (Cayman Chemical, Ann Arbor, MI), or cisplatin (Sigma Aldrich, St. Louis, MO), or with a combination of cisplatin and the sGC stimulators. Cell viability was determined by the MTT assay (Affymetrix, Cleveland, OH). For drug combination studies, synergism or additivity were assessed by calculating the combination index (CI), according to Chou/Talalay [30], wherein synergism is defined as $CI < 1$, antagonism as $CI > 1$, and additive effects as $CI = 1$. The CI values were calculated using CompuSyn software (ComboSyn, Inc., Paramus, NJ).

2.3 Flow Cytometry

Cells plated at 300,000 cells/well in 6-well plates were treated for 48h with the indicated drugs, and apoptosis was determined using FITC/Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA). Briefly, cells were incubated with Annexin V and propidium iodide for 15 min and then analyzed by flow cytometry, using a Cell Lab Quanta SC Flow Cytometer with accompanying software (Beckman Coulter). About 10,000 gated events were collected per treatment.

2.4 Real-time PCR

Total RNA was isolated using the illustra RNAspin Mini kit from GE Healthcare (Buckinghamshire, UK), and was reverse transcribed using the RT2 First Strand kit from Qiagen (Hilden, Germany). Real-time PCR was performed on 30 ng of cDNA using Absolute Blue QPCR SYBR Green ROX mix from Thermo Scientific, on an Applied Biosystems StepOnePlus real-time PCR system. Changes in EGFR and β -catenin gene expression were calculated from the cycle threshold, after correcting for cDNA amounts using β 2M expression, according to Pfaffl et al [31]. The primer pairs were as follows: EGFR (F: CTAAGATCCCGTCCATCGCC, R: GGAGCCCAGCACTTTGATCT), CTNNB1

(F: ATGACTCGAGCTCAGAGGGT, R: ATTGCACGTGTGGCAAGTTC), β 2M (F: GGCATTCCTGAAGCTGAC, R: GAATCTTTGGAGTACGCTGG)

2.5 Western blotting

Cells were treated for 24 h and then lysed on ice in RIPA buffer containing protease inhibitors (Sigma Aldrich). Protein concentrations were determined by a BCA protein assay (Life Technologies), and 30 μ g were resolved on 12% SDS-PAGE, and transferred to PVDF membranes. After blocking with 0.5% BSA, membranes were incubated in primary antibodies against EGFR, β -catenin, Bax or Bcl-2 (Cell Signaling Technologies, Danvers, MA) for 24 h, followed by several washes and incubation with HRP-conjugated secondary antibody (GE Healthcare). Blots were developed using SuperSignal West Pico chemiluminescence substrate (Life Technologies). The membranes were re-probed with β -actin antibody (Sigma Aldrich) as loading control.

2.6 Statistics

Student's t-test or ANOVA were used where appropriate. P-values ≤ 0.05 were considered significant. All experiments were repeated at 2-3 times, unless otherwise noted.

3. Results

3.1 BAY 41-2272 and YC-1 synergize with cisplatin to reduce HNSCC cell viability

We first examined the effects of the sGC stimulators YC-1 and BAY on the viability of HNSCC cells alone or in combination with cisplatin. CAL27, UM1 and UM47 cells were treated for 72h with BAY, YC-1, or cisplatin alone, or with a combination of cisplatin with BAY or YC-1. As determined by the MTT assay, YC-1, BAY or cisplatin alone dose-dependently reduced the viability of all three cell lines (Fig. 1). The combination of YC-1 or BAY with cisplatin synergistically reduced cell viability (Supplemental Table 1), as assessed by CI values < 1 , which designate a synergistic effect of the drug combination. BAY was more effective than YC-1 at lower doses, both alone and in combination with cisplatin.

3.2. BAY 41-2272 and YC-1 increase the apoptotic effects of cisplatin

We have previously shown that BAY reduced the viability of HNSCC cells primarily by inducing apoptosis, while only slightly reducing cell proliferation [22]. Here we examined whether YC-1 or BAY enhanced the apoptotic effects of cisplatin in HNSCC cells. CAL27 and UM1 cells were treated with YC-1, BAY or cisplatin alone, or with a combination of cisplatin with YC-1 or BAY for 48 hours. As determined by Annexin V staining and flow cytometric analysis, the combination of YC-1 with cisplatin increased apoptosis $\sim 70\%$ over cisplatin alone in CAL27 cells, while BAY with cisplatin increased the apoptotic effect by ~ 2 fold over either drug alone (Fig. 2A). Similar results were obtained with UM1 cells (Fig 2B).

3.3 The sGC stimulators are effective at reducing the viability of cisplatin-resistant cells

We next examined the effects of sGC stimulators on HNSCC cells with acquired cisplatin resistance. The CAL27-CisR cells were previously generated by growing the cells in

increasing doses of cisplatin over several weeks [32], while UM1-CisR cells were recently generated using the same approach. As compared to the parental cells, both cisplatin-resistant cells showed a significantly lower responsiveness to increasing doses of cisplatin (Fig 3A). In contrast, both CisR cells responded similarly to the parental cells when treated with YC-1 (Fig. 3B) or BAY (Fig. 3C), especially at the higher doses of the sGC stimulators.

3.4 BAY 41-2272 synergizes with cisplatin to decrease the viability of cisplatin-resistant cells

Next, CAL27-CisR and UM1-CisR cells were treated with combinations of cisplatin plus YC-1 or cisplatin plus BAY. The doses of YC-1 and BAY used here were the same or lower than those used in combination treatments with the parental cell lines, while the cisplatin doses were higher. As determined by the MTT assay, YC-1 in combination with cisplatin produced an additive effect on cell viability, while BAY in combination with cisplatin was synergistic (Fig 4 and Supplemental Table 2).

3.5 BAY 41-2272 suppresses the expression of EGFR and β -catenin

Previous reports have shown that drugs which increase cGMP and/or activate PKG reduce the levels of pro-survival proteins EGFR and β -catenin in some cancer cell types. We analyzed the protein levels of EGFR and β -catenin in CAL27 and UM47 cells treated with increasing doses of BAY. As determined by Western blotting, the levels of both proteins decreased dose-dependently in both cell lines (Fig. 5A and 5C). Analysis by real-time PCR revealed suppression of *EGFR* and *CTNNB1* (β -catenin) gene expression by 5 μ M BAY (Fig. 5B and 5D).

3.6 BAY 41-2272 alters the expression of survival proteins

We also examined changes in the pro-apoptotic protein Bax and anti-apoptotic Bcl-2 in response to increasing doses of BAY in CAL27 and UM47 cells. Fig. 6, top panel shows BAY-induced increase in Bax protein levels, while Bcl-2 remained relatively unchanged. Following densitometry, the change in the ratio of Bax to Bcl-2 was determined (Fig.6 lower panel). The ratio of Bax to Bcl-2 increased dose-dependently with BAY treatment in both cell lines.

4. Discussion

We are reporting that the sGC activators YC-1 and BAY 41-2272 (BAY) increase the efficacy of cisplatin to reduce viability and enhance apoptosis in HNSCC cells. Both drugs were also effective in cells with acquired cisplatin resistance (CisR). In addition, the sGC activators suppressed EGFR and β -catenin expression while increasing the levels of the pro-apoptotic protein Bax, suggesting potential mechanisms for the apoptotic and chemosensitizing effects of these drugs.

Previous studies have produced conflicting results on the consequences of activating the cGMP signaling on cancer cell survival and sensitivity to cisplatin. For example, NO donors and cGMP analogues reversed cisplatin resistance in fibrosarcoma cells [33], and PDE5 inhibitors synergized with cisplatin to reduce viability of medulloblastoma [23] and bladder

cancer cells [34]. Conversely, sGC *inhibitors* enhanced the apoptotic effect of cisplatin in non-small cell lung cancer cells [35]. A plausible explanation for this discrepancy is the differential expression of PKG isozymes, known to have disparate effects on cancer cell survival. Thus, PKG-I α has been reported to have pro-tumorigenic effects [35, 36], while PKG-I β [37, 38] and PKG-II [13] had antineoplastic effects. Our previous studies have shown that all four HNSCC cell lines examined express PKGI β and PKGII, but had no detectable levels of PKGI α transcripts [22].

Although cisplatin is a first line treatment for advanced HNSCC, intrinsic and acquired resistance present a significant hindrance to treatment efficacy [39-41]. Decreased sensitivity to cisplatin necessitates dose escalation, resulting in significant side effects, including nephrotoxicity and peripheral nerve damage [42]. In the present study we clearly demonstrate that sGC stimulators are effective at reducing the viability of HNSCC cells with acquired cisplatin resistance, and furthermore, synergize with cisplatin in these cells. Such synergistic effects were evident in diverse HNSCC cell lines, derived from different anatomical locations within the oral cavity, and with variable HPV expression. Collectively, these data suggest that sGC stimulators could be highly beneficial as additions to cisplatin treatment for HNSCC, enabling the use of lower doses of cisplatin and minimizing side effects.

Treatment with relatively low doses of BAY reduced gene expression and protein levels of the pro-survival factors EGFR and β -catenin. EGFR is overexpressed in about 90% of HNSCC cases, and correlates with worse prognosis [43]. Degradation of EGFR has been associated with the effectiveness of cisplatin in head and neck cancer, and failure of responsiveness to cisplatin has been linked to reduced EGFR degradation [28]. In human non-small cell lung cancer xenografts, an EGFR monoclonal antibody enhanced the antitumor effects of cisplatin [44].

A previous study reported that β -catenin is transcriptionally upregulated in HNSCC cells with acquired cisplatin resistance [41]. In other studies, knock down of β -catenin expression sensitized ovarian [45] and lung [46] cancer cells to cisplatin. Moreover, overexpression of PKGI β in colon cancer cells reduced β -catenin gene expression and signaling [27]. Inhibitors of PDE5, which was shown to activate the cGMP/PKG pathway, have also been found to exert pro-apoptotic effects in breast [47] and colon [48] cancer cells via inhibition of β -catenin expression and signaling downstream of PKG.

Treatment with BAY also lead to an increase expression of the pro-apoptotic protein Bax, while the pro-survival protein Bcl-2 remained unchanged. In response to apoptotic stimuli, Bax heterodimerizes with Bcl-2 to suppress its pro-survival effects. As the ratio of Bax to Bcl-2 increases, cells are more likely to undergo apoptosis [49]. The Bax:Bcl-2 ratio positively correlated with the response to cisplatin and radiation treatment in bladder cancer [50].

In summary, we have shown that NO-independent stimulators of sGC enhance the effects of cisplatin in HNSCC cells, and are also effective in cells with acquired cisplatin resistance. The mechanism behind these effects likely involves decreased expression of survival factors

and increased levels of pro-apoptotic factors. The sGC stimulator Riociguat (Adempas) was approved by the FDA for the treatment of pulmonary hypertension in 2013, and has been well tolerated with minimal side effects associated with chronic treatment. In the future, sGC stimulators could prove useful additions or alternatives to cisplatin treatment in patients with head and neck cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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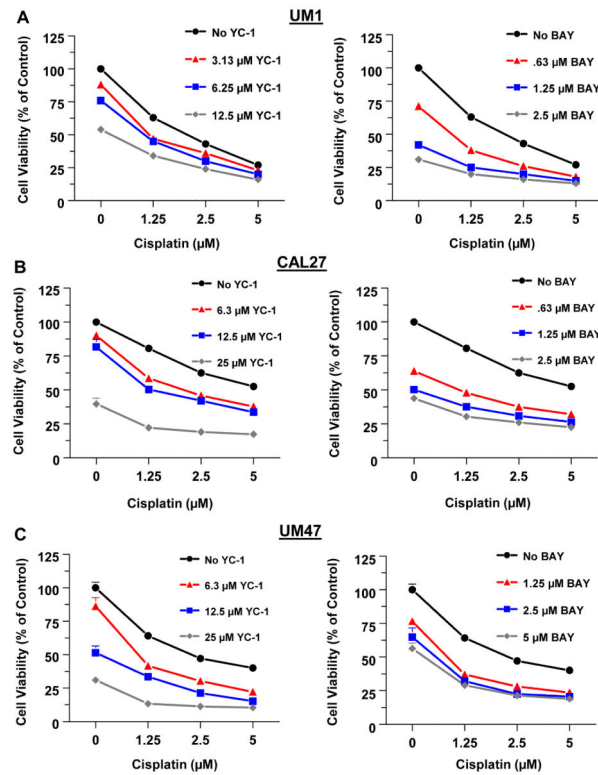
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- The sGC stimulators synergize with cisplatin to reduce viability of HNSCC cells.
- Cisplatin-induced apoptosis is enhanced by sGC stimulators.
- The sGC stimulators synergize with cisplatin in cisplatin-resistant cells.
- BAY 41-2272 lowers EGFR and β -catenin expression and increases Bax/Bcl-2 ratio.

**Fig 1.**

The sGC stimulators synergize with cisplatin to reduce the viability of HNSCC cells. The HNSCC cell lines UM1 (A), CAL27 (B) and UM47 (C) were treated with increasing doses of YC-1 (*left panels*) or BAY (*right panels*) alone, or together with the indicated doses of cisplatin for 72h. Cell viability was determined by the MTT assay. Each value is a mean±SEM of 6 replicates.

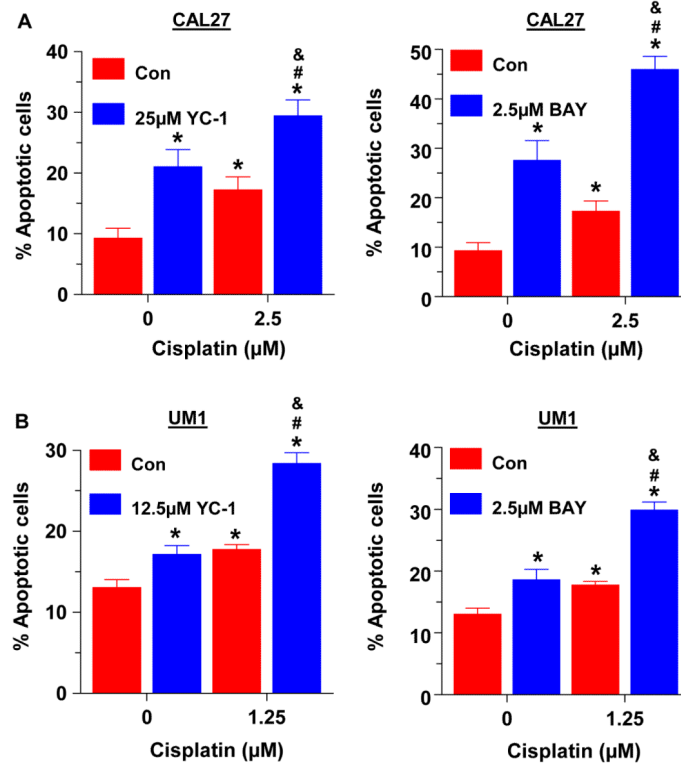


Fig 2.

The sGC stimulators enhance the apoptotic effect of cisplatin. UM1 (A) and CAL27 (B) cells were treated with YC-1 (*left panels*) or BAY (*right panels*) at the indicated doses, alone or together with cisplatin for 48h. Cells were stained with Annexin V and propidium iodide and were analyzed by flow cytometry. Each value is a mean±SEM of 3 replicates. * indicates significant ($p < 0.05$) vs. control and # indicates significant vs. single drug.

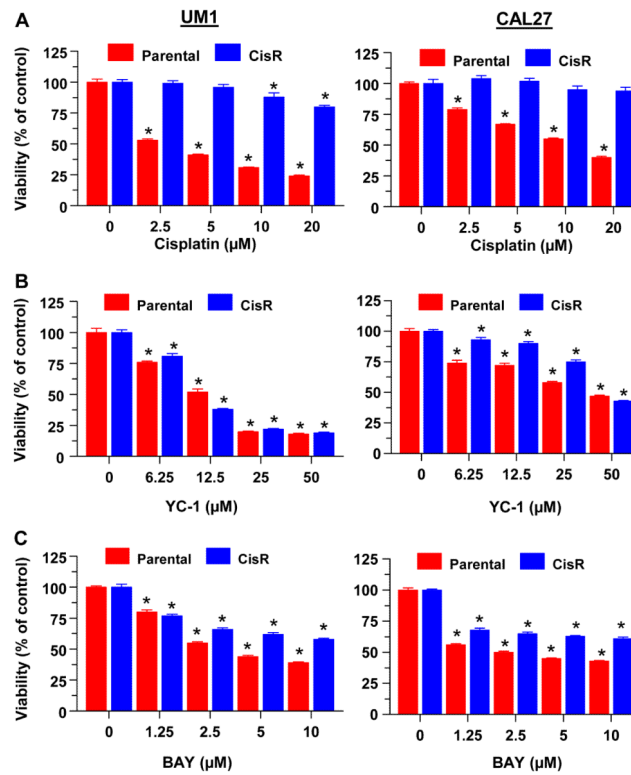
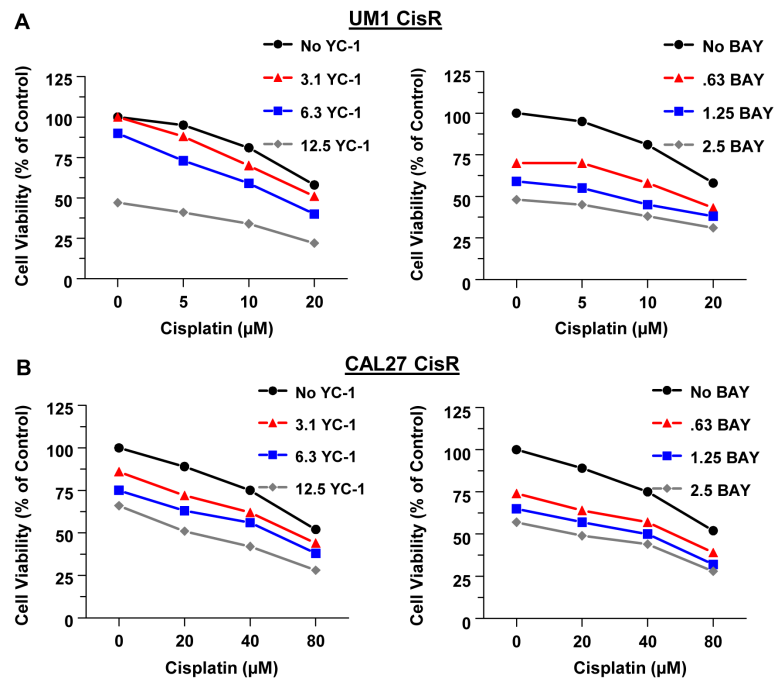
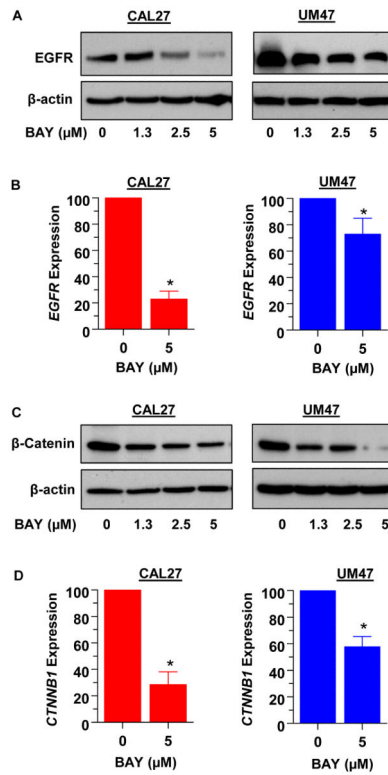


Fig 3. Cisplatin resistant HNSCC cells are sensitive to BAY and YC-1. UM1-CisR and CAL27-CisR cells and their parental lines were treated with increasing doses of cisplatin (**A**), YC-1 (**B**) or BAY (**C**) for 72h. Cell viability was determined by MTT assay. Each value is a mean \pm SEM of 6 replicates.

**Fig 4.**

The sGC stimulators synergize with cisplatin to reduce the viability of cisplatin resistant HNSCC cells. UM1-CisR (**A**) and CAL27-CisR (**B**) were treated with increasing doses of YC-1 (*left panels*) or BAY (*right panels*) alone, or with the indicated doses of cisplatin for 72h. Cell viability was determined by MTT assay. Each value is a mean \pm SEM of 6 replicates.

**Fig 5.**

Treatment with BAY reduces the expression of EGFR and β -catenin in CAL27 and UM47 cells. Cells were treated with increasing doses of BAY for 48 hours, lysed and analyzed for EGFR (A) or β -catenin (C) by Western blotting. Expression of EGFR (B) and CTNNB1 (D) mRNA was examined by real-time qPCR. Each value is a mean \pm SEM of 3 replicates.

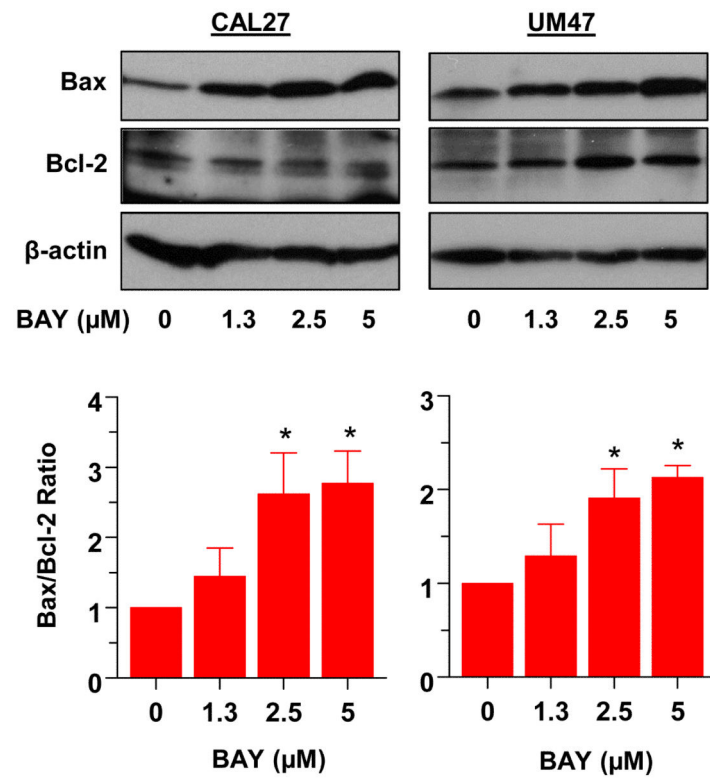


Fig 6. BAY increases the ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2 in CAL27 and UM47 cells. Cells were treated with the indicated doses of BAY for 48h, lysed and analyzed for Bax and Bcl-2 by Western blotting (*upper panel*); β -actin served as a loading control. Protein levels were quantified by densitometry, and the Bax:Bcl-2 ratio is shown in the lower panel.