

Abstract

Introduction: Cisplatin (CDDP) remains the primary chemotherapeutic agent in cases of head and neck squamous cell carcinoma (HNSCC). However, many tumors are intrinsically less sensitive to CDDP, while a large percentage of tumors that are originally sensitive develop therapeutic resistance. Thus, investigating mechanisms of resistance to identify potential actionable targets is paramount.

Methods: CDDP resistant cell lines were established and used to characterize potential mechanisms of resistance and assess viability in response to various therapeutics *in vitro*. The most promising targets were then assessed *in vivo*.

Results: Pre-clinical models of cisplatin resistance in HNSCC (HN30, HN30-R8) were generated and baseline molecular signaling characteristics were characterized by gel electrophoresis. We identified notable increases in the expression and activity of members of the MEK/ERK pathway. Homing in on this pathway, we then assessed the change in activation of these pathways in the response to increasing doses of CDDP. We noted reactivation of ERK1/2 and its downstream target, p90RSK, in HN30-R8 compared to parental HN30 at 72 hours, suggesting that this pathway may be responsible for mediating CDDP resistance. To test this hypothesis, HN30 parental cells and HN30-R8 were treated with SCH772984, a potent inhibitor of ERK1/2, and BI-D1870, an effective inhibitor of p90RSK. The CDDP resistant HN30-R8 showed greater sensitivity to SCH772984 and BI-D1870 than parental HN30 (IC₅₀ 2.3 umol/L vs 5.1 umol/L for SCH772984 and 8.8 umol/L vs 31 umol/L for BI-D1870). Based on the almost 4-fold difference in IC₅₀ for BI-D1870, we were interested in seeing if RSK overexpression alone was capable of recapitulating the CDDP resistant phenotype. To this end, the various known isoforms of p90RSK were stably overexpressed in HN30 cells and their viability with CDDP treatment was assessed. We observed that overexpression of all the isoforms, but especially p90RSK1 and p90RSK2, increased the viability at 5uM by about 40%. As the calcium-activated chloride channel, TMEM16A, has been previously shown by our group to activate ERK1/2 and correlate with CDDP resistance, we investigated if TMEM16A correlated with p90RSK expression. In a panel of HNSCC cell lines assessed via western blot analysis, we saw that the TMEM16A expression correlated proportionally with p90RSK expression and its activity. To assess the clinical utility of this discovery, we performed Chou-Talalay synergy analysis of *in vitro* efficacy of CDDP and BI-D1870 in the same panel of cell lines and noted that the combination was synergistic in high TMEM16A expressing cells and additive in low TMEM16A expressing cells. This finding was then confirmed *in vivo*.

Conclusion: In our pre-clinical model of CDDP resistance, the reduced efficacy of CDDP is mediated by p90RSK activation via ERK1/2. We have also identified that TMEM16A may serve as a biomarker for identifying tumors that may respond well to co-administration of CDDP with a p90RSK inhibitor with a synergistic effect.

Introduction

- Cisplatin (CDDP) remains central to the nonsurgical treatment of head and neck squamous cell carcinoma (HNSCC)
- Despite the significant efficacy, there remains the issue of the high rates of primary or acquired resistance
- One strategy to overcome this has been combination therapy with agents that promote HNSCC response to CDDP
- Here, we characterize a mechanism of CDDP acquired resistance via activation of p90RSK and its potential targetability**
- Further, we identify TMEM16A as a potential biomarker for combination treatment with CDDP and p90RSK inhibition**

Methods

Cell lines and reagents

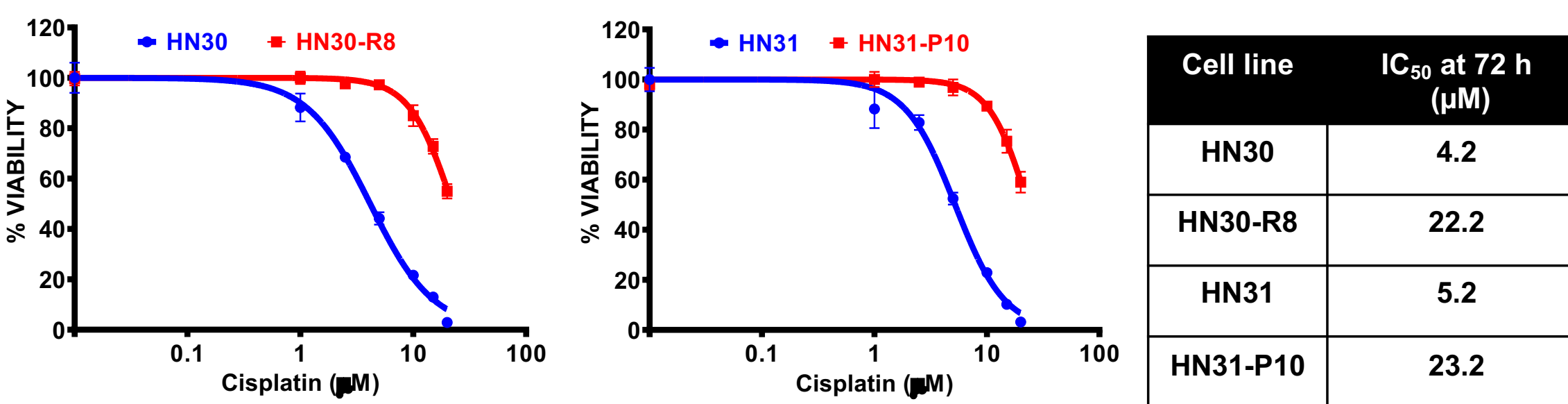
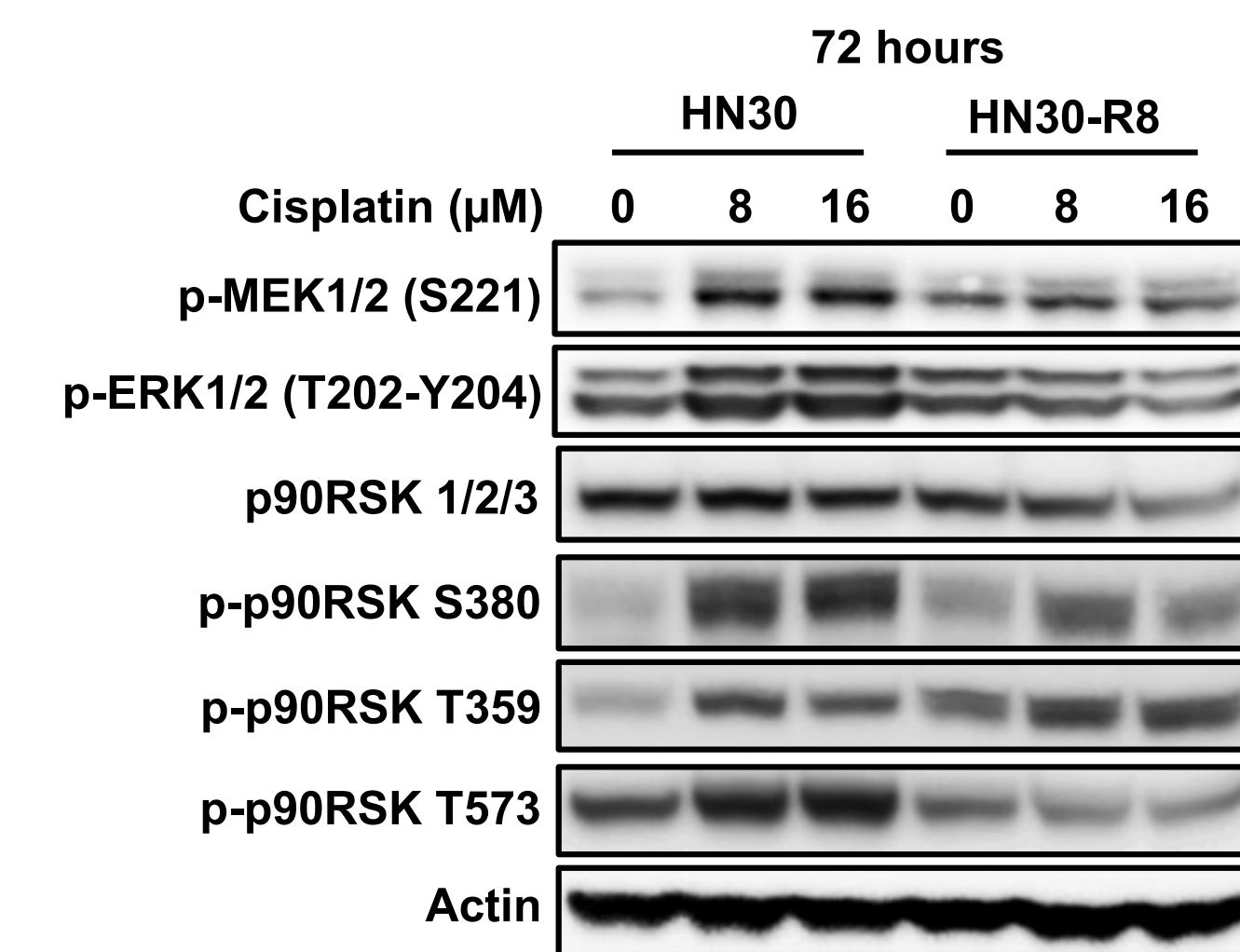
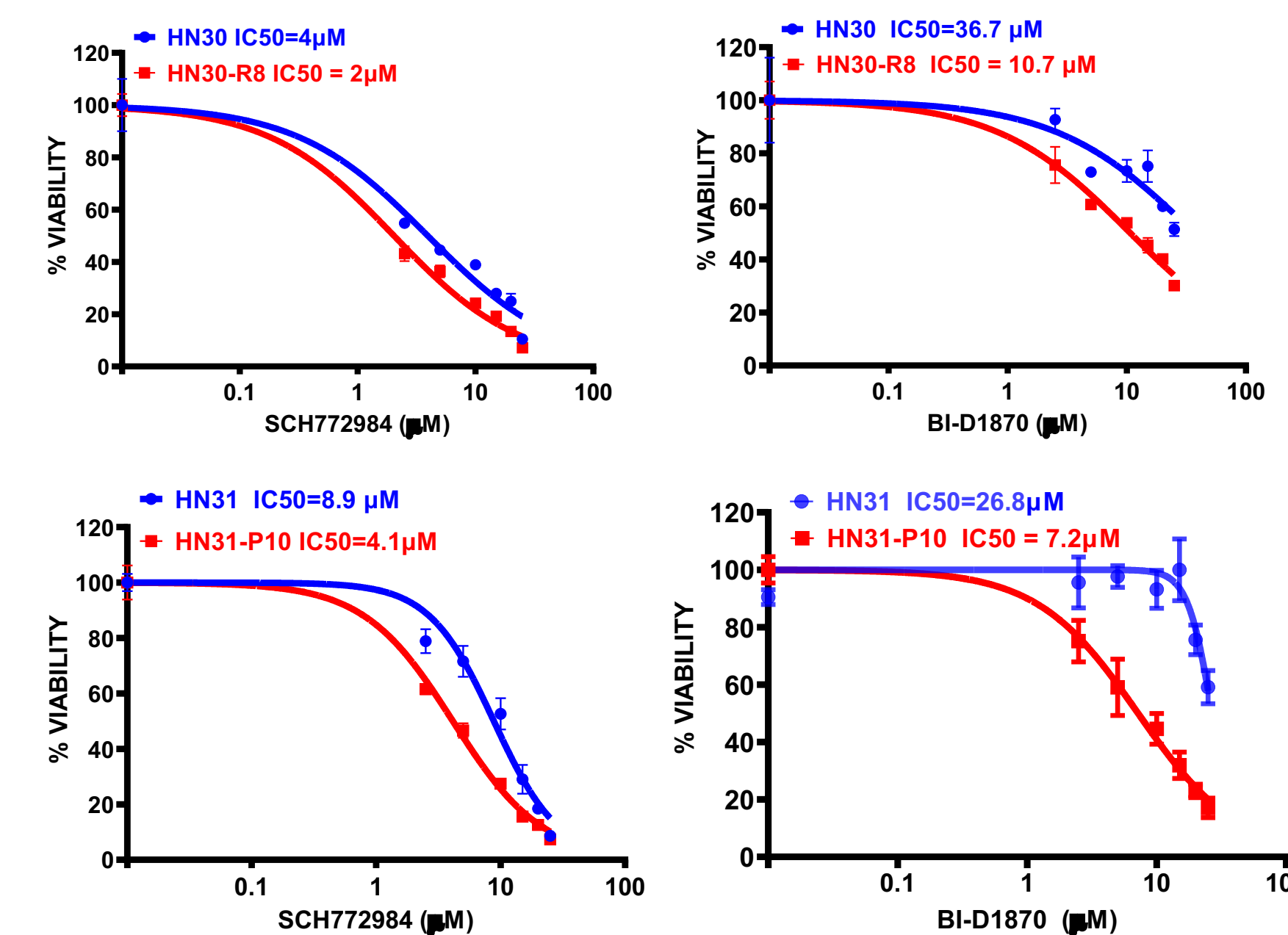
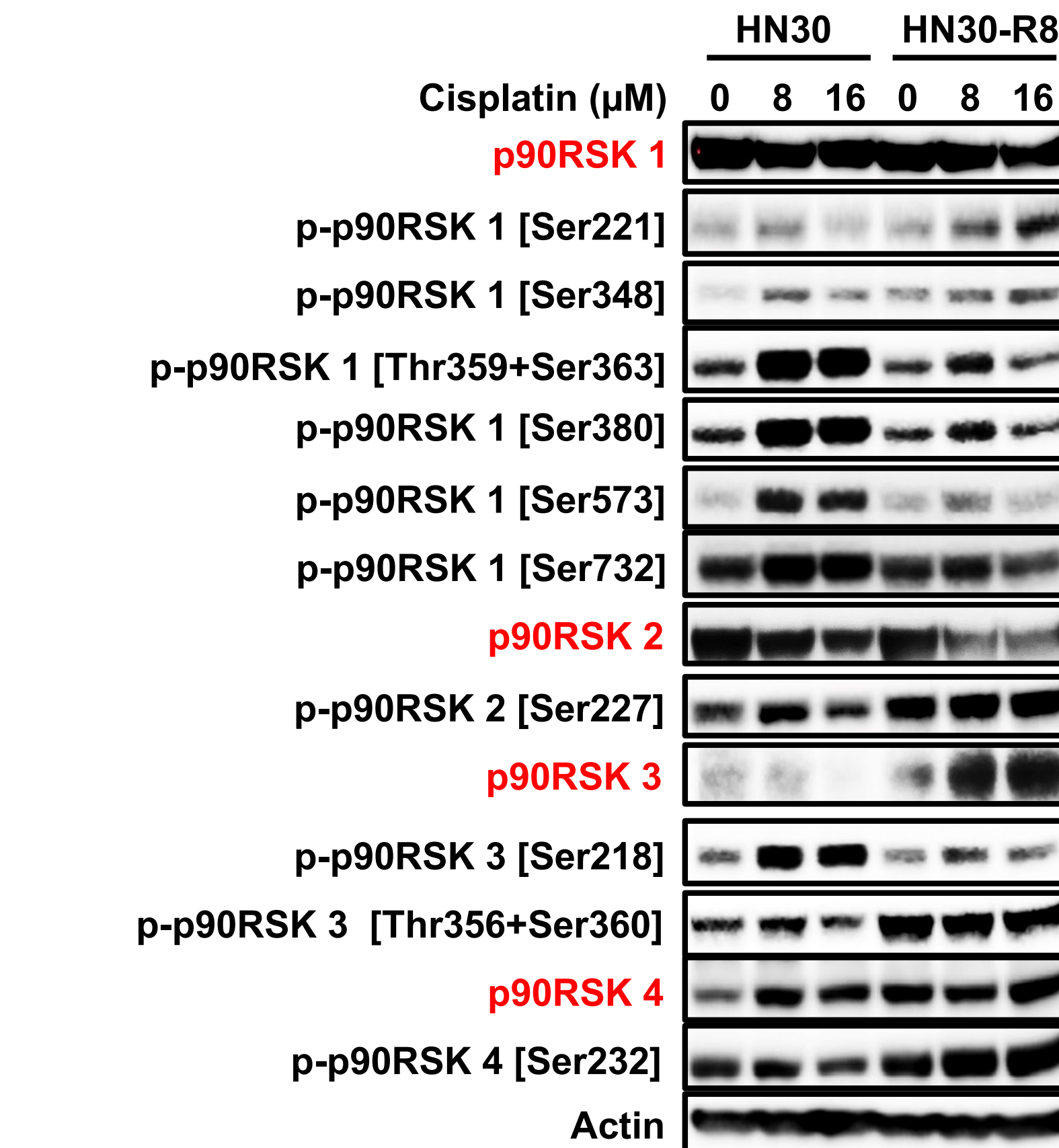
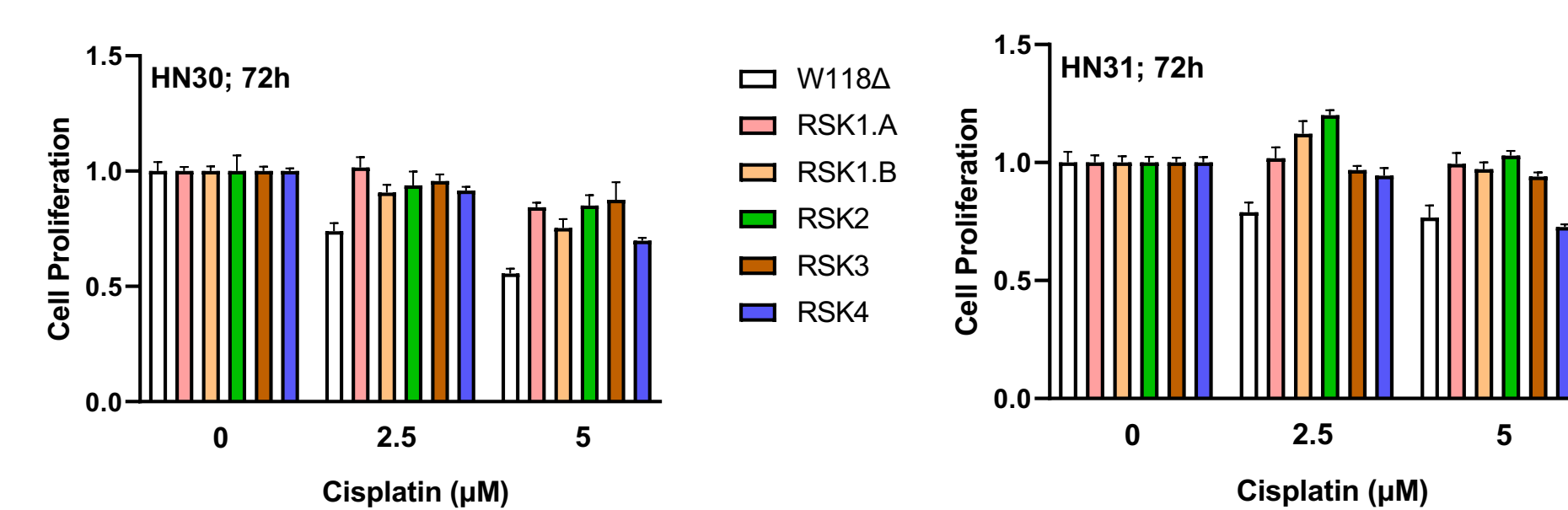
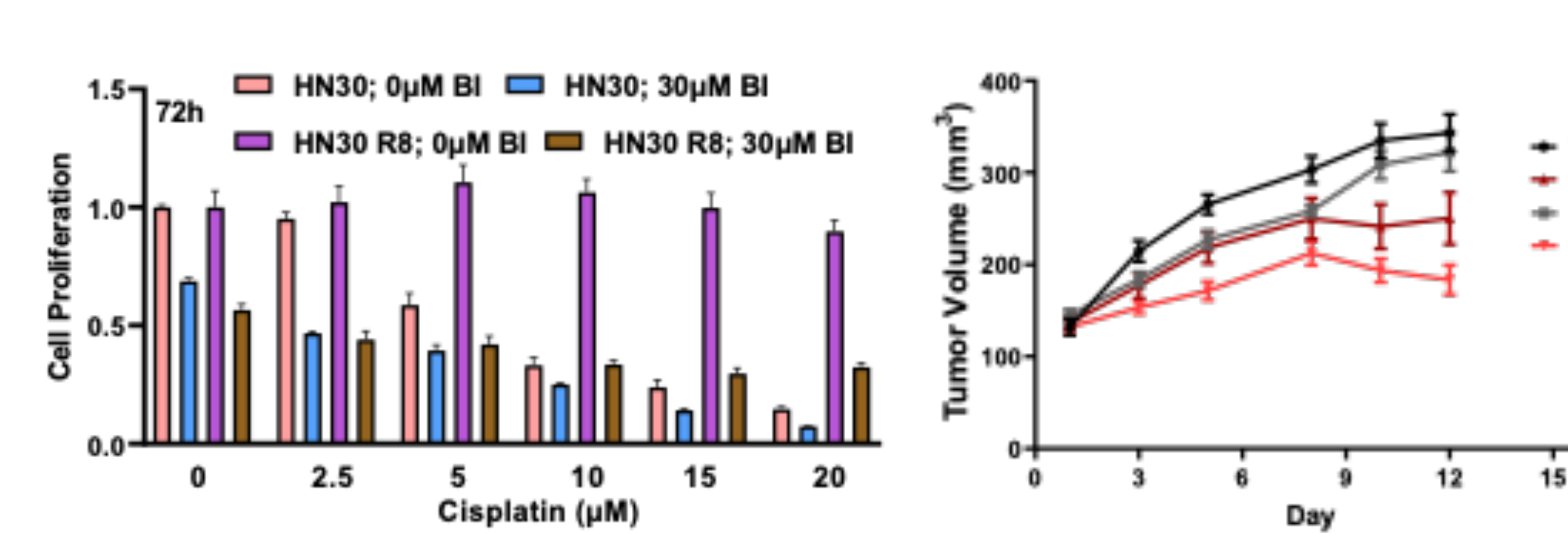
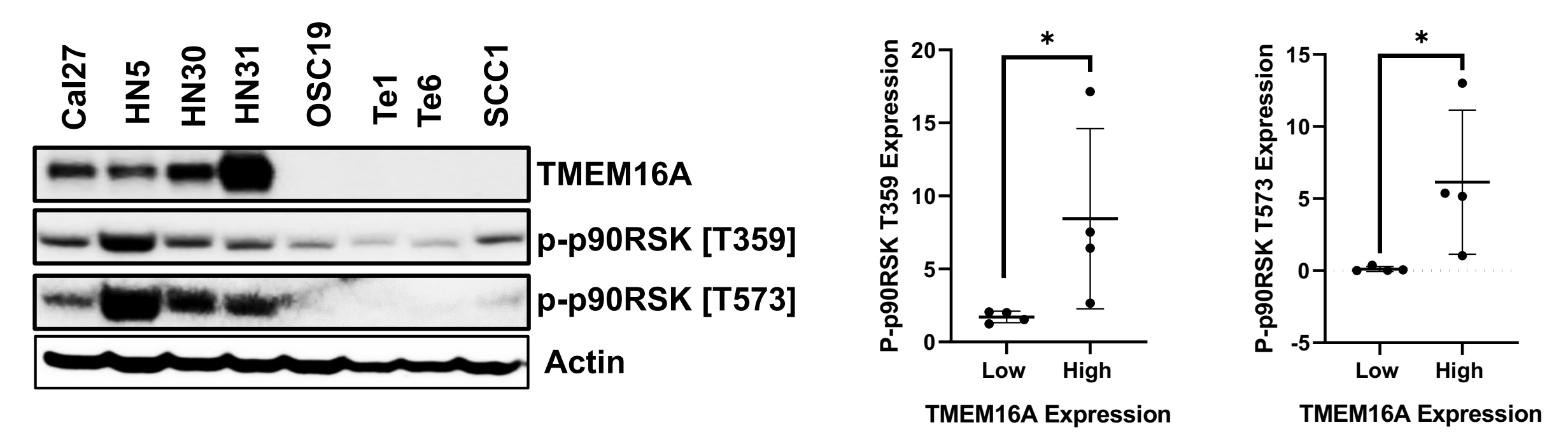
- Ten human HNSCC cell lines (HN30, HN31, HN5, Cal27, Cal33, Te1, Te6, Te9, OSC19, and SCC1) were purchased from the American Type Culture Collection (ATCC) and maintained in the laboratory following ATCC recommended growth medium.
- Cisplatin resistant cell lines HN30-R8 and HN31-P10 were generated from HN30 and HN31 parental cells respectively, by gradually increasing the cisplatin dose in culture media.
- Cisplatin was obtained from Sigma, whereas BI-D1870 and SCH772984 were purchased from Selleck Chemicals.
- Cell proliferation assays
- 2.5 to 3 X 10³ Cells/well were seeded in 96 well plates. After 24 hours, cells were treated with respective inhibitors. Cell viability at 48- and 72-hours was determined using the WST-1 Cell Proliferation Assay System (TaKaRa; Cat#MK400) following manufacturer's protocol. IC₅₀ values were calculated using Prism V8.0 (GraphPad software).
- Synergy matrix analysis was performed using Chou-Talalay method for assessing the efficacy of cisplatin and BI-D1870 in combination. Ratio of cisplatin to BI-D1870 was 1:10. Cell viability and fraction affected were assessed by WST assay following 72 hours treatment. CompuSyn web-based software was used to analyze the data.

Western blot and antibodies

- Following treatment with specific inhibitors for defined periods of times, cell pellets were collected, protein lysates were prepared, and western blotting were performed as previously described (3). All primary antibodies used in this study are listed in supplementary table. 1. Antibodies were purchased from the respective companies as mentioned in the table. Densitometry analyses were performed using ImageJ software (NIH).

In vivo experiments

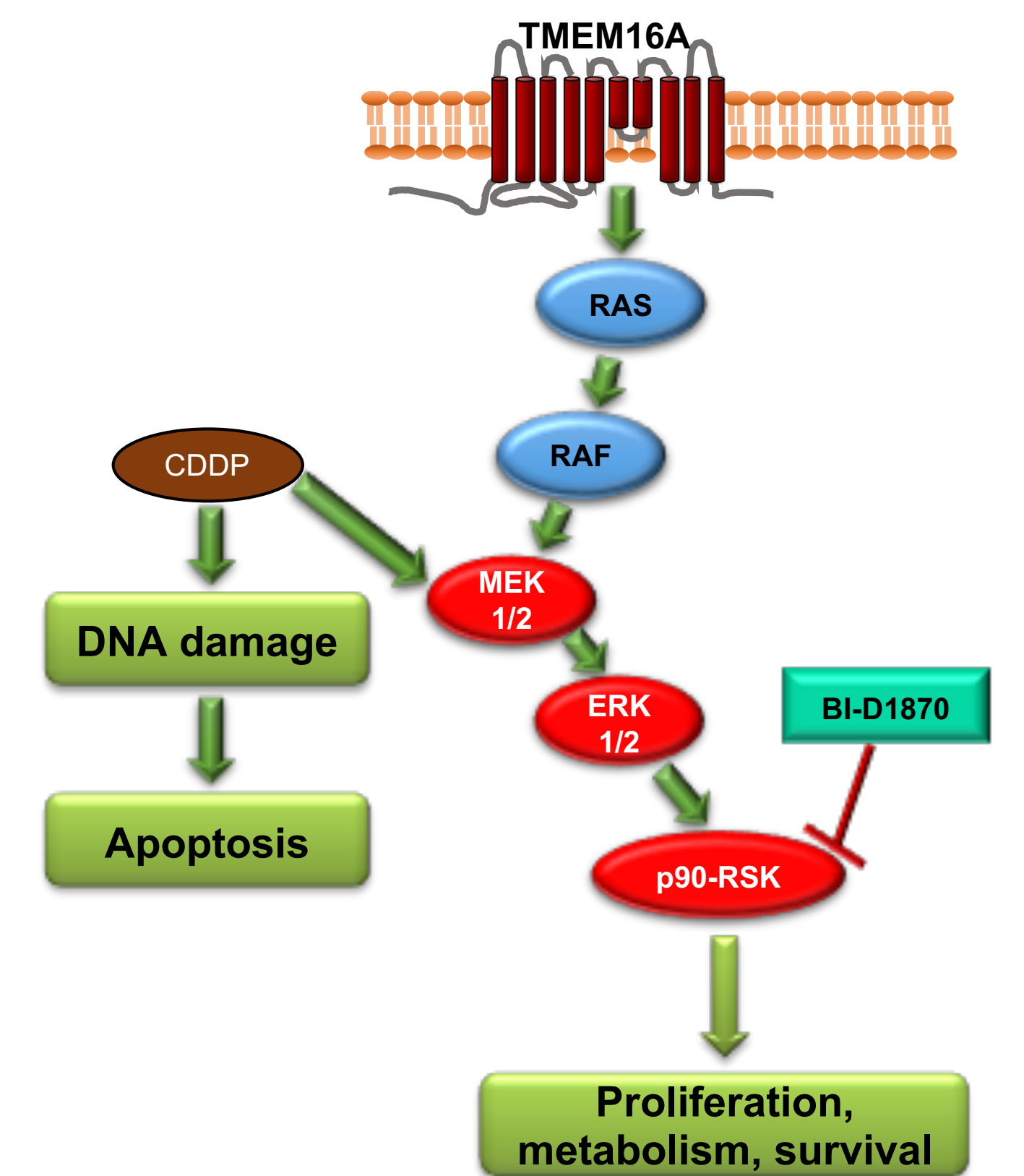
- NOD-*scid* mice were purchased from the Jackson Laboratory.
- 4 mice in each group were implanted with HN30 cells (5e6 cells per flank). Mice were treated with the appropriate drugs and euthanized after 12 days according to facility protocol.

Figure 1. Characterization of CDDP resistant HNSCC cell lines *in vitro*.**Figure 2. CDDP activates the MEK/ERK/p90RSK signaling pathway****Figure 3. CDDP resistant HNSCC cell lines are sensitive to ERK1/2 and p90RSK inhibition****Figure 4. Cisplatin resistance activates specific isoforms of p90RSK****Figure 5. p90RSK overexpression recapitulates a CDDP resistant phenotype *in vitro*****Figure 6. p90RSK inhibition improves the efficacy of CDDP *in vivo* and *in vitro*****Figure 7. TMEM16A, an activator of ERK1/2, correlates with p90RSK activation in a panel of HNSCC cell lines****Figure 8. Combination treatment with CDDP and p90RSK inhibitor is synergistic in high TMEM16A cell lines and additive in low TMEM16A cell lines**

72h	High TMEM16A expression					Low TMEM16A expression			
Cisplatin:BI (1:10)	HN30	HN5	Cal27	FaDu	Te9	OSC19	Te1	Te6	SCC1
5.5	3.69707	0.48767	1.07883	1.66123	0.68387	0.56312	0.45821	0.26482	2.09978
11.0	0.54898	0.63313	1.52651	1.40518	0.77964	0.92908	0.86021	0.75631	1.70069
22.0	0.14432	0.65117	2.3695	1.24905	1.01282	1.71031	1.54286	0.9354	1.49843
33.0	0.07838	0.64912	0.94039	0.99491	0.67237	1.59267	1.46664	1.04426	1.51512
44.0	0.01574	0.58789	0.32492	0.70836	0.32534	1.42986	1.42093	1.07743	1.38911

Conclusions

- CDDP appears to activate the MEK/ERK/p90RSK pathway which serves as a mechanism for CDDP resistance
- This pathway can be targeted with p90RSK inhibition and increases the efficacy of CDDP especially in tumors with high TMEM16A expression



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