

Introduction

Background: Cisplatin (Ct) and Cetuximab (Cx) are standard of care front-line treatments for patients with head and neck squamous cell carcinoma (HNSCC). Ct is a DNA alkylating agent and Cx is a monoclonal antibody targeting the epidermal growth factor receptor (EGFR), which is often amplified in HNSCC. Although both treatments are very effective, recurrence and metastasis are frequent due to the development of tumor resistance.

Autophagy is a homeostatic process for recycling proteins and organelles in the cell and involves the formation of specific vacuoles in the cytoplasm (1) (**Figure 1**). Several stress conditions (starvation, hypoxia, infection) are known activators of autophagy. In tumor cells, autophagy promotes their survival by facilitating adaptation to stress and by providing nutrients for their elevated metabolism. Several studies indicate that autophagy is activated in tumor cells as a treatment escape mechanism (2). Chloroquine (CQ), an FDA-approved drug for malaria and autoimmune diseases, is currently under evaluation as an autophagy inhibitor in cancer clinical trials.

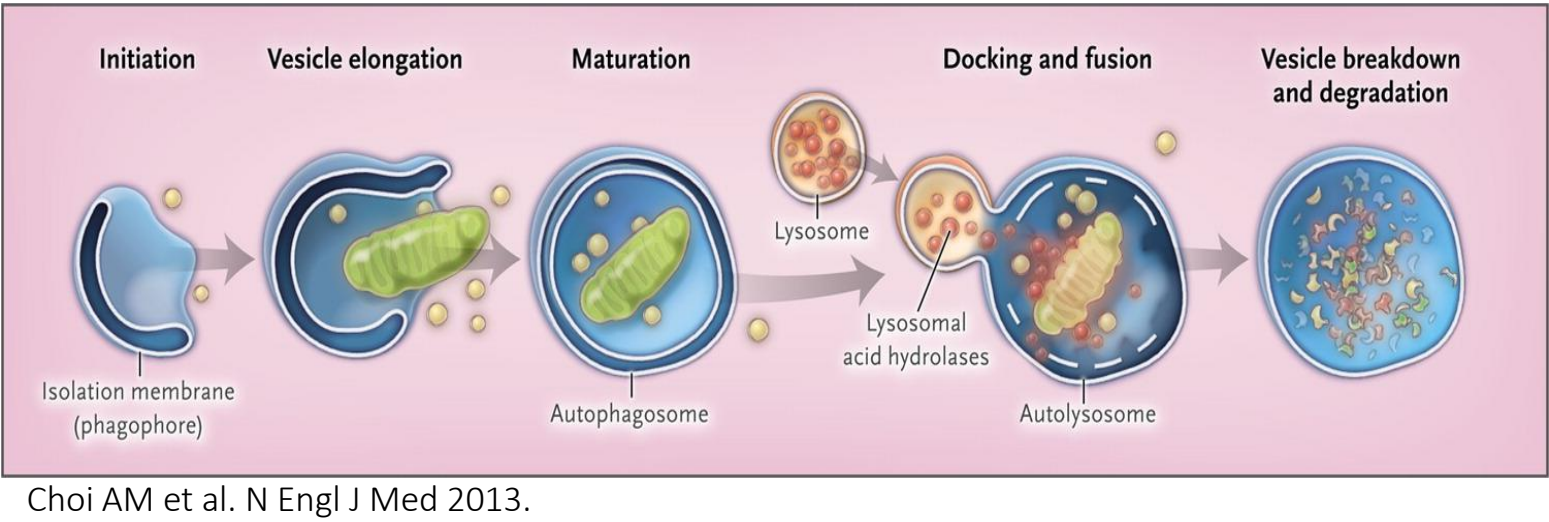


Figure 1. Phases of the Autophagic Pathway.

We recently showed that HNSCC cell lines exposed to investigational drugs Buparlisib and Omipalisib, which inhibit the phosphatidylinositol 3-kinase (PI3K) pathway, displayed an increase in autophagy (3) (**Figure 2**). PI3K inhibitors are of prime interest in HNSCC since a study by The Cancer Genome Atlas (TCGA) identified the PI3K signaling cascade as the most frequently altered pathway in HNSCC, with a gain of function in more than 75% of cases (4).

The PI3K pathway controls cell proliferation and autophagy in the cell (**Figure 3**). In our published study, we demonstrated that autophagy inhibitor CQ blocked the PI3K inhibitor-induced autophagy. PI3K inhibitors used in conjunction with CQ acted in synergy to reduce HNSCC cell proliferation. These results suggest that autophagy inhibition may enhance the efficacy of therapeutics in HNSCC. In particular, EGFR is upstream of the PI3K pathway and Cx was shown to induce autophagy in some HNSCC cell lines (5). For Ct, several resistance mechanisms have been identified, which include autophagy activation (6).

Objective: To evaluate the efficacy of Chloroquine combined with Cisplatin or Cetuximab in reducing HNSCC cell proliferation.

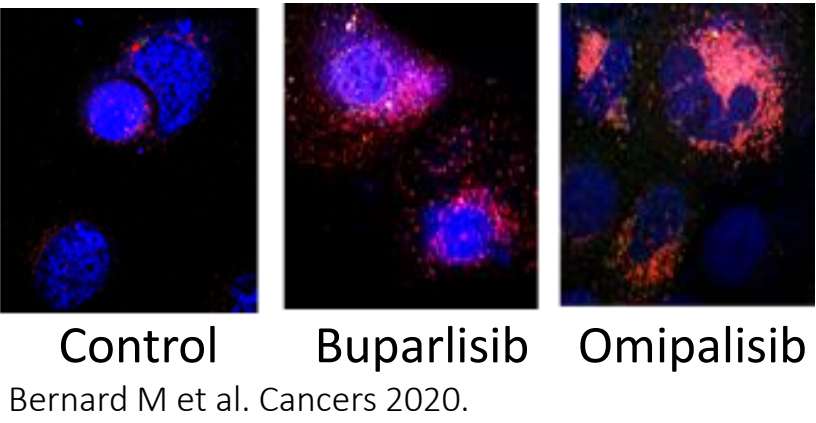


Figure 2. Autophagosomes increase with PI3K inhibitors.

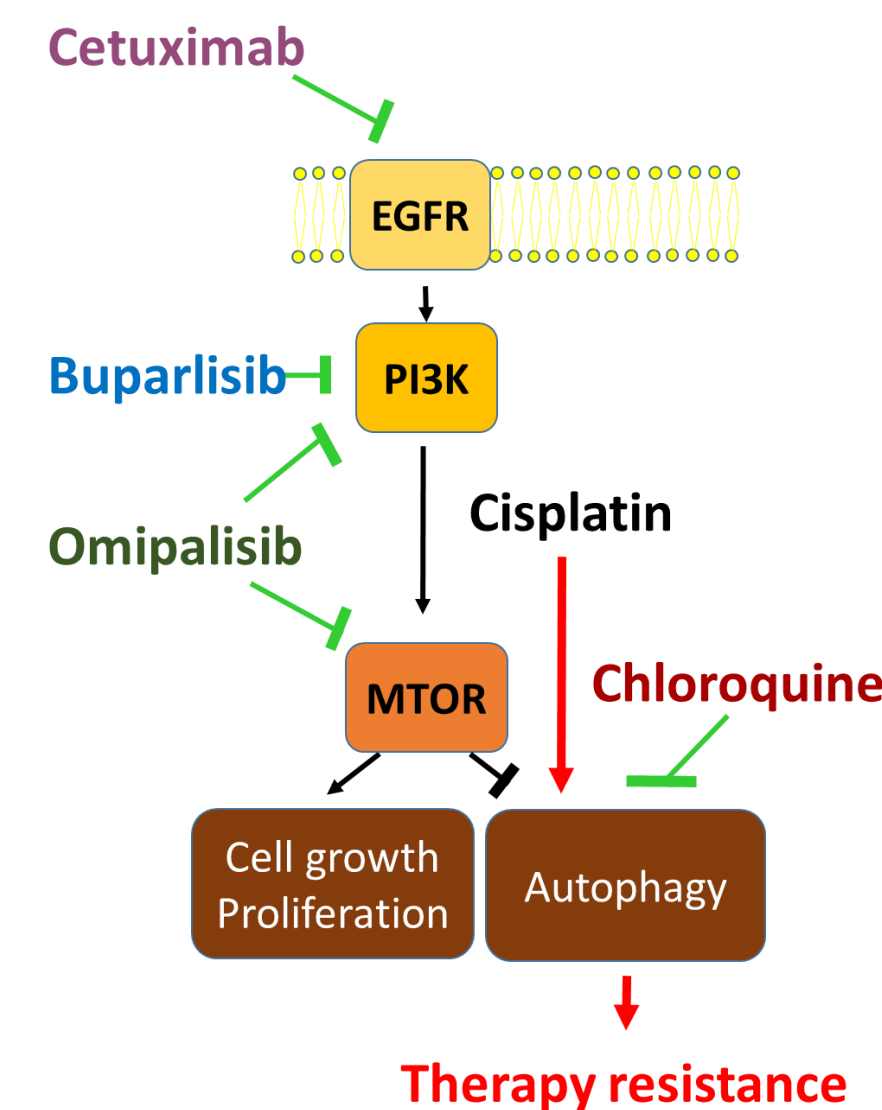


Figure 3. PI3K pathway.

Methods



Figure 4. Real-time imaging.

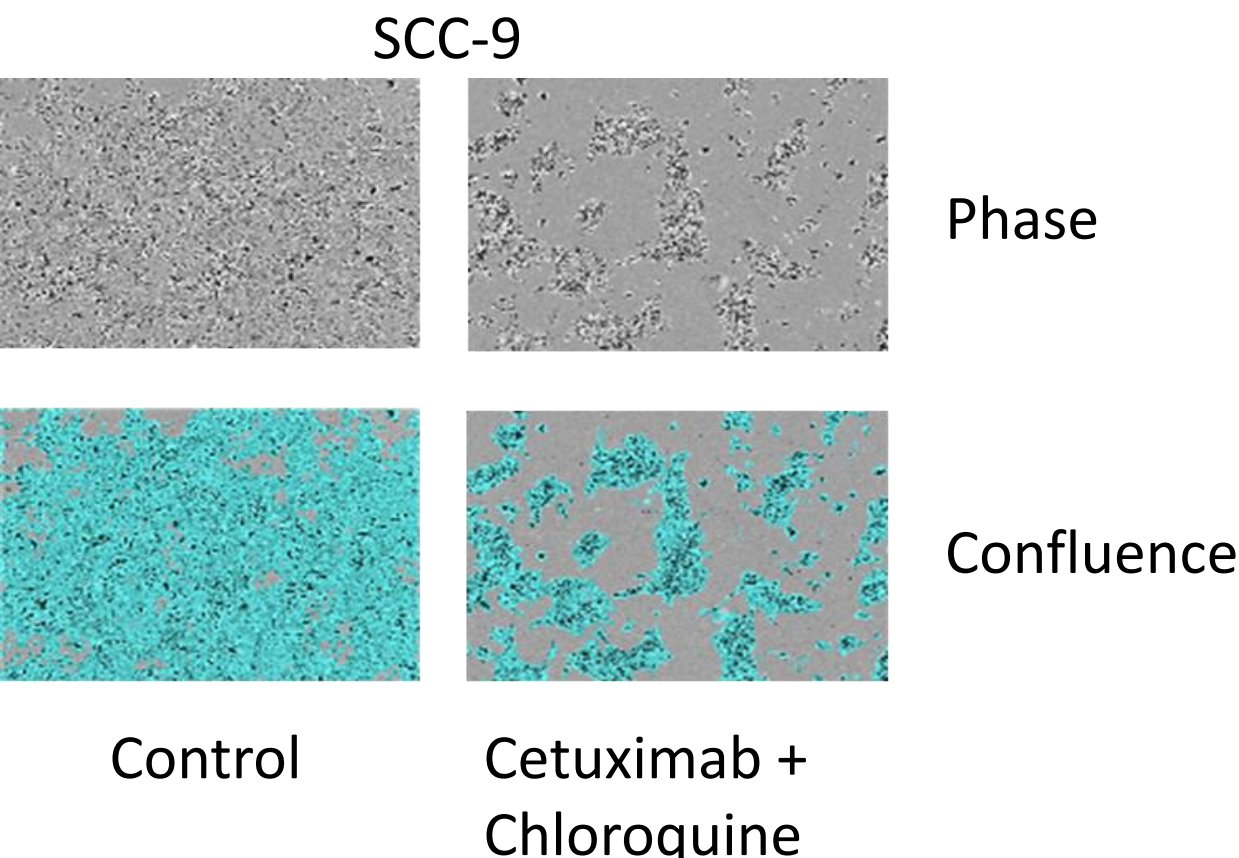


Figure 5. Confluence evaluation.

HNSCC cell lines of the tongue (SCC-4, SCC-9, SCC-25 from ATCC) and hypopharynx (FaDu from ATCC, UM-SCC-22A from EMD Millipore) were grown in DMEM with 10% FBS, 1% penicillin/streptomycin and 1X non-essential amino acids. Cells were seeded in 96-well plates and allowed to adhere overnight. The following day, the vehicle or autophagy inhibitor (CQ) or Cetuximab (Cx) or Cisplatin (Ct) or combination was added in triplicates. Proliferation assays consisted of longitudinal imaging of the cell layer (**Figure 4**) and confluence evaluation (**Figure 5**) with the IncuCyte S3 system (Essen Bioscience, Ann Arbor, MI, USA). Phase contrast images of the full well were acquired at 4 hours intervals for 3 days at 4X magnification. Proliferation curves were generated using the confluence evaluation algorithm of the IncuCyte S3 software, and values were normalized to confluence at time = 0 hour. Area under the curve (AUC) with baseline correction was calculated using GraphPad v8. AUC results were expressed as % proliferation versus control and formatted in a matrix for analysis with SynergyFinder software (7) (<https://synergyfinder.fimm.fi/synergy>). A Bliss synergy score over 1 indicates synergy (**Figure 6**).

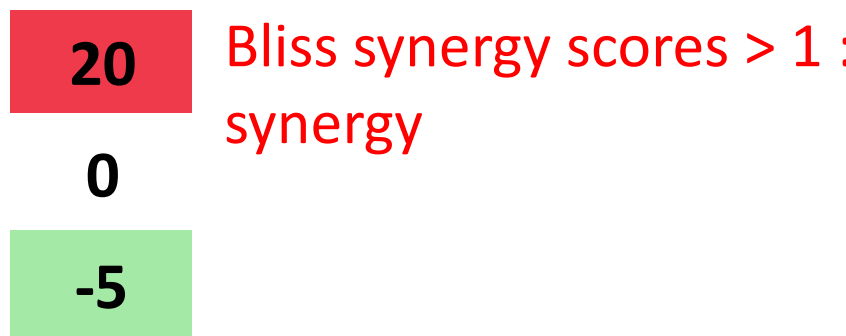
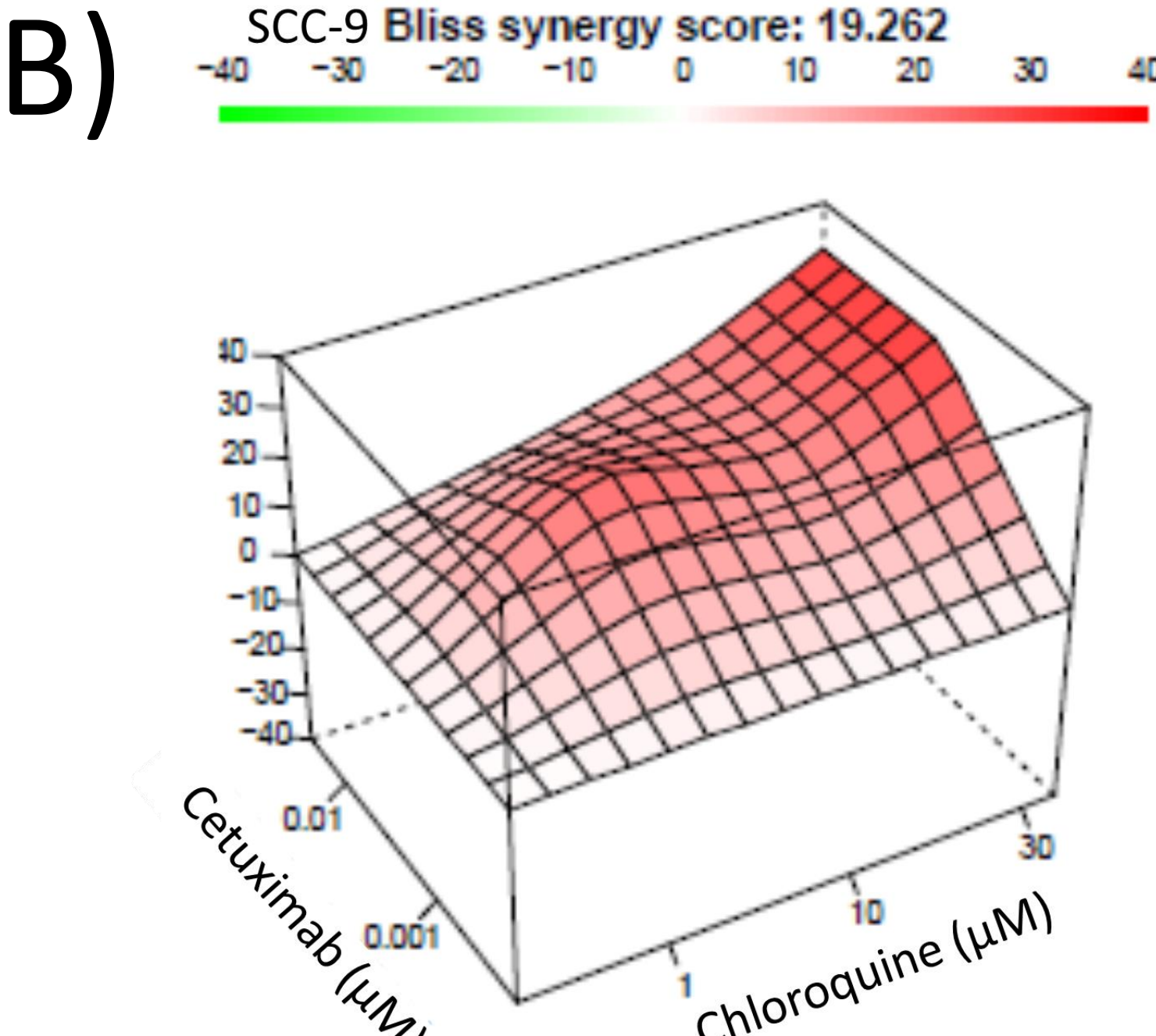
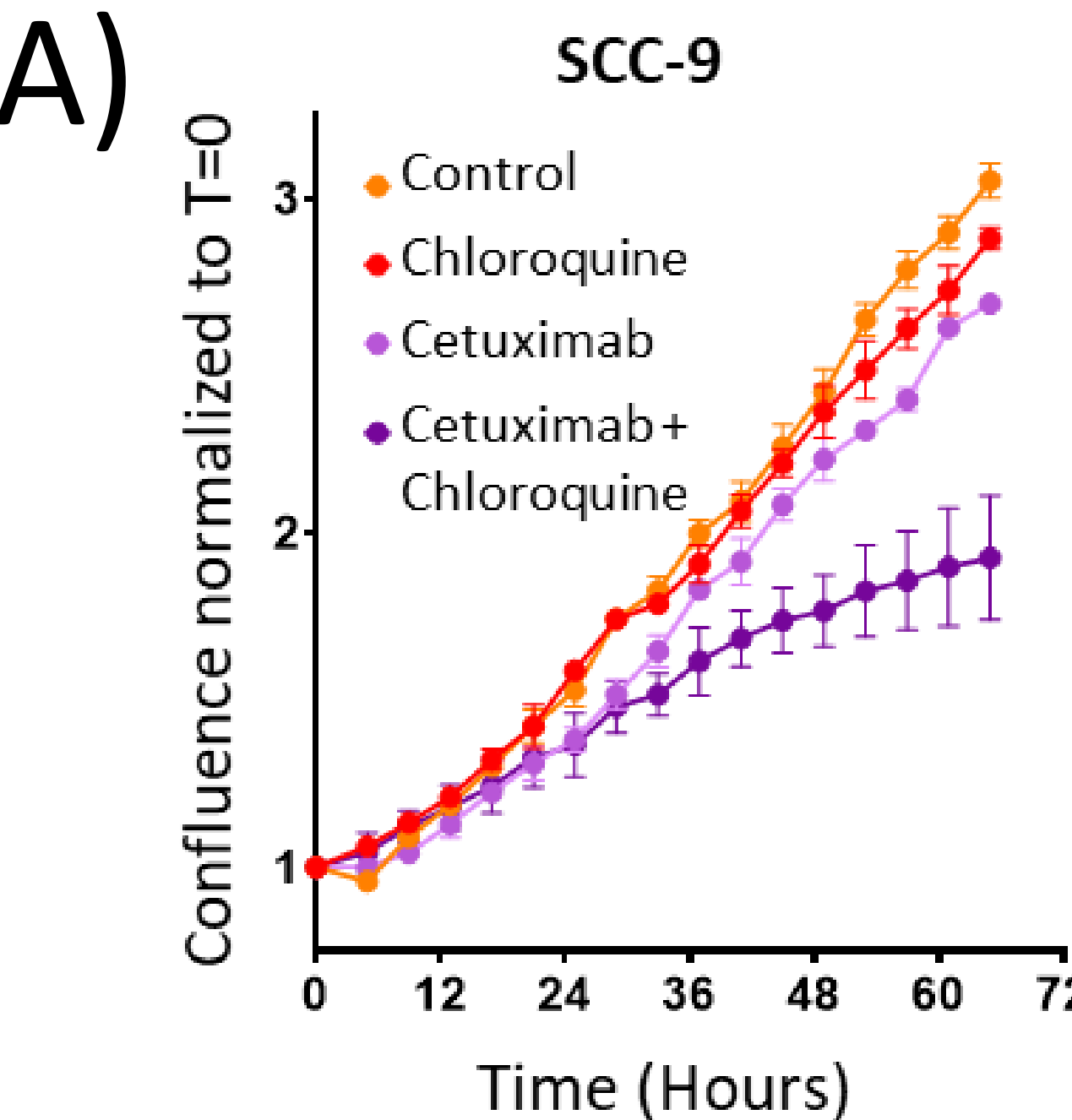


Figure 6. Color code for Bliss score.

Results



Cell line	Cx+CQ	Ct+CQ
SCC-9	19,26	3,94
FaDu	18,15	1,01
SCC-25	14,44	8,59
UM-SCC-22A	11,41	-3,14
SCC-4	9,68	-0,67

Figure 7. Cetuximab (Cx) and Cisplatin (Ct) work in synergy with autophagy inhibitor Chloroquine (CQ) to decrease proliferation of HNSCC cell lines. (A) Real-time cell proliferation of SCC-9 captured by IncuCyte S3 for 3 days with vehicle (Control), 10 nM Cx and $\pm$ 10 μ M CQ. Data are the mean of triplicates $\pm$ SEM normalized to time 0 hour and are representative of three independent experiments. **(B) Visualization of synergy score** with three-dimensional diagram representing Bliss score (z) versus Cx concentration (y) and CQ concentration (x). SCC-9 cell line was incubated with different concentrations of CQ or Cx alone or in combination (1, 10, 30 μ M CQ; 1 or 10 nM Cx). Real-time proliferation was recorded for 3 days before AUC determination and Bliss score evaluation. **(C) Bliss synergy scores** for treatment combinations: Cx+CQ or Ct+CQ. HNSCC cell lines were incubated with different concentrations of the inhibitors alone or in combination (1, 10, 30 μ M CQ; 1 or 10 nM Cx; 1 or 5 μ M Ct). Real-time proliferation was recorded for 3 days before AUC determination and Bliss score evaluation.

Conclusion and Perspectives

Conclusion: The CQ + Cx combination demonstrated synergy in all five HNSCC cell lines, and the CQ + Ct combination showed a synergistic effect in three of the cell lines. These results are in line with our published work, which showed that PI3K inhibitors used in conjunction with CQ demonstrated a synergism that reduced HNSCC cell proliferation. Altogether, our results suggest that autophagy is an important target in HNSCC and that the combination of autophagy inhibition with standard of care therapy represents a promising therapeutic strategy in HNSCC.

Perspectives:

- Study autophagy and EGFR signaling pathway inhibition at the molecular level.
- Establish a xenograft mouse model to study the effect of combination treatments *in vivo*.
- Establish an *ex vivo* model (Lab-On-A-Chip) (8, 9) (**Figure 8**) to study new drugs, new treatment strategies or to test available therapies on a patient's own tumor specimen before chemotherapy treatment. We anticipate that the development of precision medicine will help to determine better therapeutic choices for improved treatment outcomes.

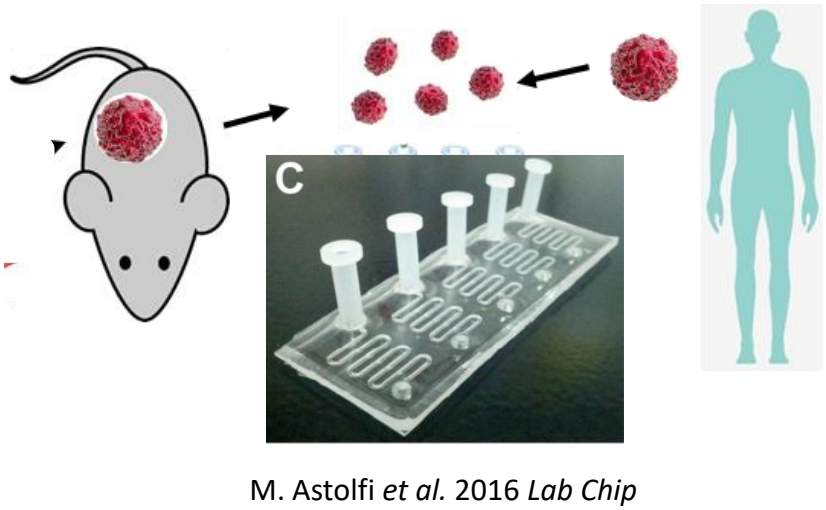


Figure 8. Lab-On-A-Chip microfluidic device for *ex vivo* incubation of micro-dissected tumor tissue.

References

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