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# Inhibition of autophagy with chloroquine is effective in melanoma

Michael E. Egger, MD,[a,b] Justin S. Huang, BS,[b,c] Wenyuan Yin, BS,[b,c] Kelly M. McMasters, MD, PhD,[a,b] and Lacey R. McNally, PhD[b,c,*]

[a] Hiram C. Polk Jr MD Department of Surgery, University of Louisville, Louisville, Kentucky
[b] James Graham Brown Cancer Center, Louisville, Kentucky
[c] Department of Medicine, University of Louisville, Louisville, Kentucky

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ABSTRACT

*Background:* Cancer cells adapt to the stress resulting from accelerated cell growth and a lack of nutrients by activation of the autophagy pathway. Two proteins that allow cell growth in the face of metabolic stress and hypoxia are hypoxia-inducible factor-1α (HIF-1α) and heat shock protein 90 (Hsp 90). We hypothesize that chloroquine (CQ), an antimalarial drug that inhibits autophagosome function, in combination with either echinomycin, a HIF-1α inhibitor, or 17-dimethylaminoethylamino-17-dimethoxygeldanamycin (17-DMAG), an Hsp 90 inhibitor, will result in cytotoxicity in melanoma.

*Materials and methods:* Multiple human melanoma cell lines (BRAF wild-type and mutant) were tested *in vitro* with CQ in combination with echinomycin or 17-DMAG. These treatments were performed in hypoxic (5% $O_2$) and normoxic (18% $O_2$) conditions. Mechanism of action was determined through Western blot of autophagy-associated proteins HIF-1α and Hsp 90.

*Results:* Chloroquine, echinomycin, and 17-DMAG each induced cytotoxicity in multiple human melanoma cell lines, in both normoxia and hypoxia. Chloroquine combined with echinomycin achieved synergistic cytotoxicity under hypoxic conditions in multiple melanoma cell lines (BRAF wild-type and mutant). Western blot analysis indicated that echinomycin reduced HIF-1α levels, both alone and in combination with CQ. Changes in LC3 flux indicated inhibition of autophagy at the level of the autophagosome by CQ therapy.

*Conclusions:* Targeting autophagy with the antimalarial drug CQ may be an effective cancer therapy in melanoma. Sensitivity to chloroquine is independent of BRAF mutational status. Combining CQ with the HIF-1α inhibitor echinomycin improves cytotoxicity in hypoxic conditions.

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## 1. Introduction

Outcomes for patients with cutaneous melanoma are quite good if the diagnosis is made early and aggressive surgical resection is performed prior to metastatic spread; however standard systemic therapies for metastatic disease have dismal complete response rates of approximately 5% [1]. Newer agents targeting T-cell regulation (ipilimumab) or the

* *Corresponding author.* Department of Medicine, University of Louisville, 505 S. Hancock Street, CTR Rm 307, Louisville, KY 40202. Tel.: +1 502 852 2288; fax: +1 502 852 2123.
E-mail address: lrmcna01@louisville.edu (L.R. McNally).
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V600E BRAF mutation (vemurafenib) have shown promising improvements in response rates, but most patients will progress or develop resistance [2–4]. The broad applicability of vemurafenib is limited, as only 50% of melanoma patients carry the V600E BRAF mutation [5,6]. Novel therapies are needed with improved durable response rates and wider target populations to treat metastatic melanoma.

A potential target for cancer therapy is autophagy inhibition. Autophagy is a normal cellular response to stress in which organelles, cytoplasm, proteins, and metabolic byproducts are degraded; this process is crucial to the survival and growth of apoptosis-deficient cancer cells [7]. Autophagic processes protect the cellular genome and preserve limited metabolic resources in cancer cells in which dysregulated growth processes produce metabolic stress [8,9]. Inhibition of autophagy may disrupt this compensatory process, resulting in the accumulation of metabolic stress products and induction of cell death [7,10,11].

In this study, we evaluate chloroquine, 17-dimethylaminoethylamino-17dimethoxygeldanamycin (17-DMAG), and echinomycin to target cell stress response pathways. The antimalarial drug chloroquine (CQ) disrupts autophagy by inhibiting the acidification of the lysosomes that fuse with the autophagosomes, thereby preventing the degradation of metabolic stress products and inducing apoptosis [12–15]. Chloroquine-mediated inhibition of autophagy has been demonstrated in melanoma [16–18]. Heat shock protein 90 (Hsp 90) is an important molecular chaperone involved in protein folding and a regulator protein involved in the cellular response to metabolic stress that may be a useful target in cancer cells [19,20]. 17-DMAG, an analogue of geldanamycin, is an inhibitor of Hsp 90. Hypoxia-inducible factor-1 (HIF-1) is a regulatory transcription factor involved in the hypoxic stress response and a potential anticancer target [21]. Echinomycin is an inhibitor of HIF-1$\alpha$ DNA binding activity [22]. Using a combination approach targeting both autophagy and the hypoxic stress response, we hypothesize that CQ is an effective antimelanoma therapy *in vitro*, particularly in hypoxic conditions found in melanoma. Combining CQ with either 17-DMAG or echinomycin may enhance the cytotoxicity of CQ therapy in normoxic and hypoxic conditions.

## 2. Materials and methods

### 2.1. Cell lines and culture conditions

Human melanoma cells lines SK-MEL-2 and A375 were obtained from the American Type Culture Collection (Rockville, MD). Human melanoma cell line DM6 was kindly provided by D.S. Tyler (Duke University, Durham, NC). SK-MEL-2 and A375 were cultured in Dulbecco's modified Eagle medium, and DM6 cells were cultured in Iscove's modified Dulbecco medium. All media were supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% L-glutamine. Cell culture reagents were purchased from Gibco (Life Technologies, Grand Island, NY). Cells were cultured in 37°C, 5% $CO_2$ incubators. Both DM6 and A375 melanoma cell lines have the V600E BRAF mutation, whereas SK-MEL-2 is BRAF wild-type (personal communication with D.S. Tyler regarding DM6 cell line) [23].

### 2.2. Reagents

Chloroquine(Thermo Fisher, Waltham, MA), 17-DMAG, and echinomycin were purchased from Acros (Thermo Fisher Scientific, NJ), Tocris Bioscience (Bristol, United Kingdom), and Cayman Chemical (Ann Arbor, MI), respectively. Chloroquine stock solution of 10 mM was prepared with phosphate-buffered saline. Echinomycin and 17-DMAG stock solutions were prepared in dimethyl sulfoxide (10 μM and 1 mM, respectively). Reagents were stored at −20°C (echinomycin) or −80°C (chloroquine and 17-DMAG) until use.

### 2.3. Cytotoxicity determination

Human melanoma cell lines were plated in 96-well black plates at a concentration of $2 \times 10^3$ cells/well. Twenty-four hours following plating and incubation under normoxic conditions, cells were treated with increasing concentrations of CQ, 17-DMAG, and echinomycin in quadruplicate, then incubated under normoxic or hypoxic conditions. Normoxic conditions were held in standard laboratory incubators at 37°C, 5% $CO_2$, and 18% (ambient) $O_2$ levels. Hypoxic conditions were maintained in an Oasis hypoxic incubator (Caron, Marietta, OH) at 37°C, 5% $CO_2$, and 5% $O_2$. Control cells were left untreated. Cell viability was determined at 24 and 48 h following treatment using the ATPlite assay system and read on a TopCount microplate counter (PerkinElmer, Waltham, MA). Percent cell viability was determined by dividing the treated cells' luminescence counts by the corresponding control cells. Combination therapies with CQ and 17-DMAG and CQ and echinomycin were evaluated in a similar manner. Combination therapy results were analyzed with Calcusyn version 2.1 (Biosoft, Cambridge, United Kingdom) and synergistic effects were determined by calculating the combination indices by the method of Chou-Talalay (combination index $< 1.0$ indicates synergism) [24]. The nonparametric Kruskal-Wallis test was used to compare differences in viability across treatments. Statistical analysis was performed using SAS 9.3 (SAS, Cary, NC).

### 2.4. Protein analysis

Human melanoma cells A375 and DM6 were plated in 6-well plates and allowed to attach for 24 h in normoxic conditions. Cells were then left untreated (control), or treated with CQ alone (50 μM), echinomycin alone (50 pM), or CQ and echinomycin together. Treated cells were then incubated under either normoxic (18% $O_2$) or hypoxic (5% $O_2$) conditions as described above. Whole-cell protein lysates were collected 6 and 12 h following treatment using sodium orthovanadate (Sigma-Aldrich, St. Louis, MO) immediately upon removal from hypoxic or normoxic incubators. NuPAGE 4–12% Bis Tris gels were used for protein electrophoresis (Life Technologies, Grand Island, NY). Transfer to nitrocellulose membranes was performed using the iBLOT gel transfer device from Invitrogen (Life Technologies). Odyssey blocking buffer was used for Western blotting (LI-COR, Lincoln, NE). Primary antibodies for Western blotting were as follows: HIF-1$\alpha$, LC3, and Atg 7

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