

Electrochemotherapy with bleomycin is effective in BRAF mutated melanoma cells and interacts with BRAF inhibitors

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Background. The aim of the study was to explore the effectiveness of electrochemotherapy (ECT) during the treatment of melanoma patients with BRAF inhibitors. Its effectiveness was tested on BRAF mutated and non-mutated melanoma cells *in vitro* and in combination with BRAF inhibitors.

Materials and methods. ECT with bleomycin was performed on two human melanoma cell lines, with (SK-MEL-28) or without (CHL-1) BRAF V600E mutation. Cell survival was determined using clonogenic assay to determine the effectiveness of ECT in melanoma cells of different mutation status. Furthermore, the effectiveness of ECT in concomitant treatment with BRAF inhibitor vemurafenib was also determined in BRAF mutated cells SK-MEL-28 with clonogenic assay.

Results. The survival of BRAF V600E mutated melanoma cells was even lower than non-mutated cells, indicating that ECT is effective regardless of the mutational status of melanoma cells. Furthermore, the synergistic interaction between vemurafenib and ECT with bleomycin was demonstrated in the BRAF V600E mutated melanoma cells.

Conclusions. The effectiveness of ECT in BRAF mutated melanoma cells as well as potentiation of its effectiveness during the treatment with vemurafenib *in vitro* implies on clinical applicability of ECT in melanoma patients with BRAF mutation and/or during the treatment with BRAF inhibitors.

Key words: electrochemotherapy; BRAF inhibitors; vemurafenib; melanoma

Introduction

Recently, there have been great advances in the treatment of metastatic melanoma, including targeted therapy with BRAF and MEK inhibitors, and immunomodulation either with anti-CTLA-4 or anti-PD-1 or anti-PD-L1 antibodies.¹⁻⁵

BRAF inhibitors are based on the fact that 50% of melanoma tumors harbor BRAF V600 mutations, which cause increased activation of MAP kinase signaling pathway that results in melanoma cell proliferation.⁶ Inhibitors of BRAF V600 mutated melanoma, such as vemurafenib and dabrafenib, increase progression-free survival and overall survival^{1,7,8}, but unfortunately the resistance mecha-

nisms usually appear to re-establish the signaling pathway and the disease progresses in the matter of months after the start of treatment.⁹

For the progressive disease after treatment with BRAF inhibitors, other locoregional treatments are usually needed to control tumor relapses. Electrochemotherapy (ECT) provides this approach. It is a combined local treatment in which locally-applied high voltage electric pulses are used to facilitate the uptake of non-permeant chemotherapeutic drugs.¹⁰ It is used mainly for the treatment of cutaneous and subcutaneous metastases of different tumor histology, with a complete and objective response rate of 59.4% and 84.1%, respectively.¹¹ Specifically for melanoma, the com-

plete and objective response rates of 56.8% and 80.6% were obtained.¹¹

The unexplored question is whether ECT is effective in BRAF mutated melanoma cells, and whether ECT is effective as concomitant treatment to therapy with BRAF inhibitors. In the first reported case regarding treatment of a patient undergoing dabrafenib treatment, ECT proved to be effective on progressing tumor nodules.¹² Based on this report, studies on effectiveness of ECT with bleomycin in BRAF V600 mutated, compared to non-mutated melanoma cells are warranted. Besides this aim, we also investigated whether the concomitant vemurafenib treatment of cells can affect the effectiveness of ECT in melanoma cells *in vitro*.

Materials and methods

Cell lines and culturing

Human melanoma cell line SK-MEL-28 (American Type Culture Collection (ATCC), Manassas, VA, USA) with BRAF V600E mutation was cultivated in an Advanced MEM medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 5% fetal bovine serum (FBS, Gibco), 10 mM L-glutamine (GlutaMAX, Gibco), 100 U/ml penicillin (Grünenthal, Aachen, Germany) and 50 µg/ml gentamicin (Krka, Novo mesto, Slovenia) in a 5% CO₂ humidified incubator at 37°C.

Human melanoma cell line CHL-1 (ATCC) without BRAF V600 mutations was cultured in an Advanced RPMI 1640 medium (Gibco), supplemented with 5% FBS, 10 mM L-glutamine, 100 U/ml penicillin and 50 µg/ml gentamicin in a 5% CO₂ humidified incubator at 37°C.

Electrochemotherapy (ECT)

Melanoma cells were grown as a monolayer until they reached at least 80% confluence. The medium was removed and the cells were washed with phosphate-buffered saline (PBS, Merck Millipore, Darmstadt, Germany). After that, cells were detached from the surface with 0.25% trypsin/EDTA in Hank's buffer (Gibco). After detachment, trypsin was inactivated with an equal amount of cell culture medium with FBS and cells were collected and centrifuged. For electroporation, 88 µl of cell suspension (25 × 10⁶ cells/ml) was prepared in electroporation buffer (125 mM sucrose, 10 mM K₂HPO₄, 2.5 mM KH₂PO₄, 2 mM MgCl₂ × 6H₂O) at 4°C. Cell suspension was mixed with 22 µl of different stock concentrations of bleomycin (Bleomycin medac,

Medac, Wedel, Germany) to reach a final concentration of 1.4 × 10⁻¹² M, 1.4 × 10⁻¹¹ M, 1.4 × 10⁻¹⁰ M, 1.4 × 10⁻⁹ M, 1.4 × 10⁻⁸ M, 1.4 × 10⁻⁷ M, 1.4 × 10⁻⁶ M. Out of 110 µl of mixture, 50 µl served as a control for bleomycin treatment and other 50 µl was pipetted between two stainless steel parallel plate electrodes (2 mm apart) and 8 square-wave electric pulses (amplitude over distance ratio of 1300 V/cm, duration of 100 µs and frequency of 1 Hz) were applied. Electric pulses were generated with the electric pulse generator GT-01 (Faculty of Electrical Engineering, University of Ljubljana, Ljubljana, Slovenia). The cells were incubated 5 min after electroporation at room temperature, and then cell culture medium was added. Afterwards, clonogenic assay was performed. Experimental groups were denoted: BLM (different concentrations of bleomycin); BLM + EP (ECT with bleomycin of different concentrations). Each group was normalized to the control group of same treatment regimen with 0 M bleomycin (Ctrl, EP).

Clonogenic assay

After the treatment, cells were plated in 6 cm petri dishes with 4 ml of culture medium for clonogenic assay. The number of plated cells was in a range of 300-4000 cells, based on predicted cytotoxicity of the treatment. Colonies were formed after 12 and 8 days for SK-MEL-28 and CHL-1, respectively. After the colonies were formed, they were fixed and stained with crystal violet solution (Sigma-Aldrich, St. Louis, MO, USA) and counted. The colonies containing less than 50 cells were disregarded. Plating efficiency was calculated for each experimental group as the ratio between counted colonies and the number of plated cells. Plating efficiency of each treatment group was normalized to the untreated cells group, representing cell survival. IC₉₀ value was determined (drug concentration required to reduce cell survival for 90%).

Vemurafenib sensitivity

Cell lines were tested for their sensitivity to vemurafenib treatment in order to confirm the selectivity of vemurafenib on BRAF V600E mutated cells and to determine the optimal concentration for the combination treatment. Cells were detached from cell culture dishes and prepared for clonogenic assay. Vemurafenib (MedChem Express, Monmouth Junction, NJ, USA) was obtained in 10 mM DMSO solution and was added to 6 cm culture dishes with 4 ml of culture medium to reach a final concentra-

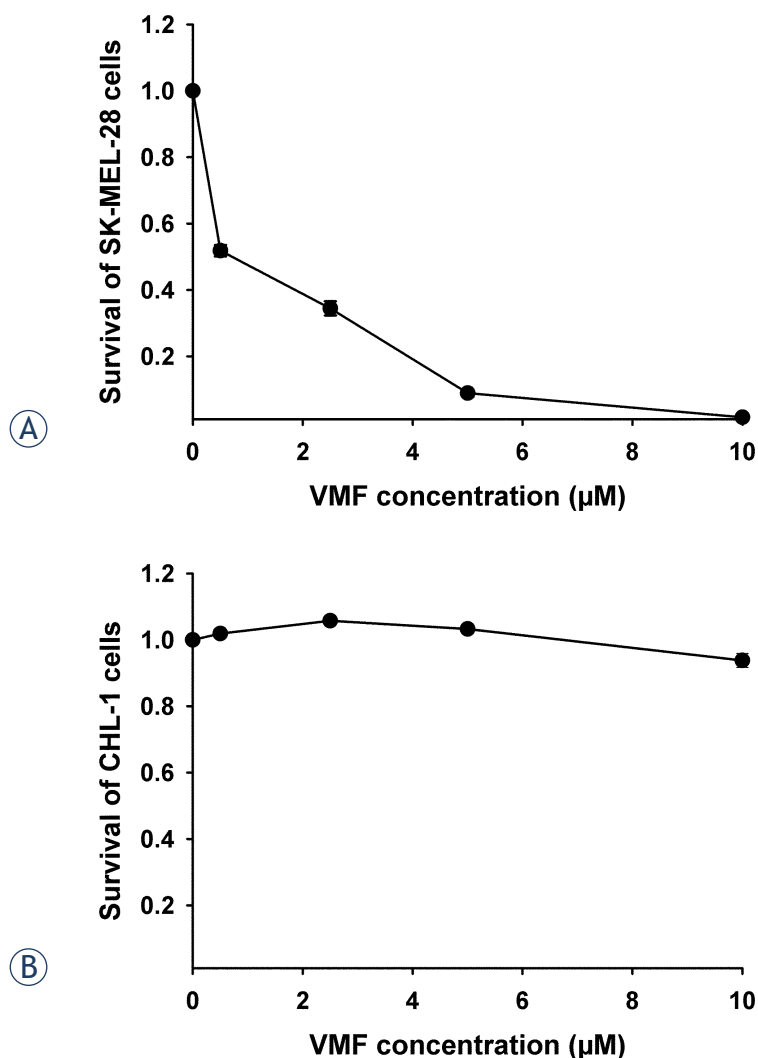


FIGURE 1. Survival of melanoma cells after vemurafenib (VMF) treatment. Survival of (A) BRAF V600E mutated melanoma cells SK-MEL-28 and (B) non-mutated melanoma cells CHL-1.

tion of 0.5 µM, 2.5 µM, 5 µM and 10 µM. The final amount of DMSO in the cell culture medium represented 20000x – 1000x dilution and was not toxic to the cells. Afterwards, cells were cultured in a 5% CO₂ humidified incubator at 37°C until the formation of colonies, which were counted and analyzed as described. Each group was normalized to the control group with 0 µM vemurafenib.

Combination of ECT and vemurafenib

ECT was performed as described. After that, cells were plated in 6 cm petri dishes for clonogenic assay and vemurafenib was added in each dish to reach a final concentration of 0.5 µM. Afterwards, cells were cultured in a 5% CO₂ humidified in-

cubator at 37°C until the formation of colonies, which were counted and analyzed as described. Experimental groups were denoted: BLM (different concentrations of bleomycin); BLM + EP (ECT with bleomycin of different concentrations); BLM + VMF (bleomycin of different concentrations and addition of 0.5 µM vemurafenib), BLM + EP + VMF (ECT with bleomycin of different concentrations and addition of 0.5 µM vemurafenib). Each group was normalized to the control group of same treatment regimen with 0 M bleomycin (Ctrl, EP, Ctrl + VMF, EP + VMF).

Statistical analysis

The mode of interaction between the treatments with independent mode of action was calculated at the level of IC₉₀ by the method developed by Spector.¹³ For the analysis and graphical representation, SigmaPlot Software (version 12.0, Systat Software, London, UK) was used.

Results

Sensitivity of BRAF V600E mutated melanoma cells to vemurafenib

Vemurafenib treatment was tested for selective cytotoxicity to BRAF V600E mutated cells. Selectivity of BRAF inhibitor vemurafenib was confirmed on SK-MEL-28 BRAF V600E mutated melanoma cells. Cell survival was reduced with increasing vemurafenib concentration with IC₉₀ 5 µM (Figure 1A). In contrast, vemurafenib treatment did not reduce the survival of BRAF V600E non-mutated cell line CHL-1. The cell survival was higher than 94%, also with the highest concentration of vemurafenib used (Figure 1B). These results also provided bases for the combined vemurafenib and ECT treatment of cells, the concentration of 0.5 µM was selected, which reduced cell survival to 50%.

BRAF V600E mutated melanoma cells are more sensitive to ECT than non-mutated melanoma cells

The effectiveness of ECT was tested on BRAF V600E mutated melanoma cells (SK-MEL-28) and non-mutated melanoma cells (CHL-1). ECT effectively reduced survival of both cell lines with IC₉₀ 3.8 × 10⁻¹⁰ M and 7.7 × 10⁻¹⁰ M for SK-MEL-28 and CHL-1, respectively. ECT was even more effective on BRAF V600E mutated SK-MEL-28 cells that required 2 times lower concentration of BLM

at IC_{90} compared to non-mutated cells CHL-1 (Figure 2), confirming that ECT is an effective method for treatment of also BRAF V600E mutated melanoma cells.

Concomitant vemurafenib treatment increased ECT effectiveness

Concomitant treatment with ECT and vemurafenib was simulated *in vitro* on cells in the way, that ECT treated cells were seeded into dishes containing 0.5 μ M vemurafenib. The vemurafenib treatment decreased survival of SK-MEL-28 cells for 50%. If the effect of the vemurafenib was eliminated (normalized to control groups with added vemurafenib for groups BLM + VMF and BLM + EP + VMF), as shown in Figure 3, then an increased effectiveness of ECT was observed on BRAF mutated SK-MEL-28 cells. A 4.5 times lower concentration of BLM was needed at the IC_{90} for cells treated with vemurafenib (BLM + EP + VMF; IC_{90} value 8.5×10^{-11} M) compared to cells without vemurafenib treatment (BLM + EP; IC_{90} value 3.8×10^{-10} M). The potentiation was more than additive (Figure 3), in fact, according to the method developed by Spector *et al.* was synergistic.¹³

Discussion

This study demonstrates the effectiveness of ECT with bleomycin on BRAF V600E mutated melanoma cells. The effectiveness was higher to that on BRAF non-mutated cells. Furthermore, an interaction of ECT and vemurafenib treatment was observed in BRAF mutated melanoma cells, indicating on more than additive or synergistic effectiveness.

BRAF inhibitors provide a clear benefit to patients with disseminated disease. The effect is mediated by inhibition of cell proliferation through the inhibition of the MAPK pathway.¹⁴ Patients often present with multiple metastases, that may not all respond to the treatment either due to their bigger size or due to the development of the resistance to BRAF inhibitors. In such cases additional therapy is needed, that has proven effectiveness also in BRAF mutated melanoma cells. Our study demonstrates that ECT with BLM is as effective, or even more effective on BRAF mutated, compared to non-mutated melanoma cells, although the exact biological mechanism still needs to be explored. The data support the recent observation on melanoma patient undergoing dabrafenib treatment

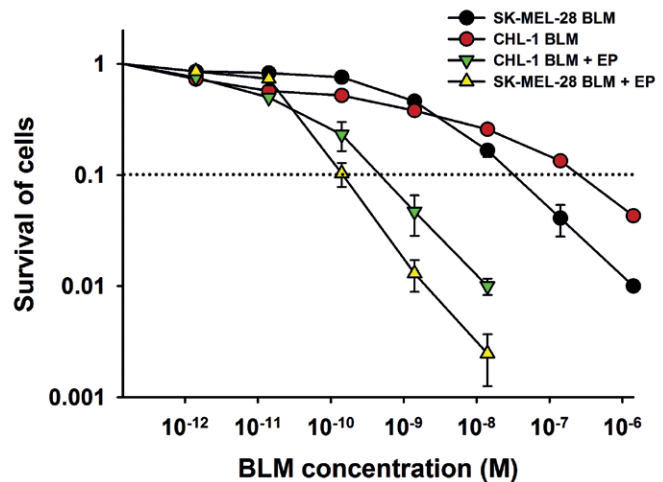


FIGURE 2. Survival of BRAF V600E mutated SK-MEL-28 melanoma cells and non-mutated CHL-1 cells after ECT with bleomycin. BLM (bleomycin); BLM + EP (ECT with bleomycin). Dotted line represents the IC_{90} value.

where some nodules were treated with ECT. ECT proved effective even on tumor nodules, which were in progression during the dabrafenib treatment¹², which indicates that it could be effective also on dabrafenib resistant tumor clones.

The other aspect that was clarified is that ECT can be successfully implemented also during the treatment with BRAF inhibitors. The *in vitro* results demonstrated that vemurafenib and ECT treatment has synergistic effectiveness. This is of clinical importance, since the *in vitro* data indicate that there is no need to wait for the discontinuation of

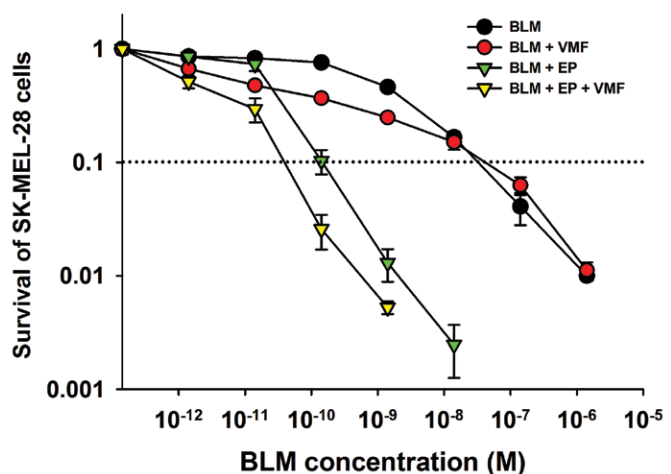


FIGURE 3. Survival of BRAF V600E mutated SK-MEL-28 melanoma cells after concomitant treatment with ECT and vemurafenib. BLM (bleomycin); BLM + VMF (bleomycin and 0.5 μ M vemurafenib); BLM + EP (ECT with bleomycin); BLM + EP + VMF (ECT with bleomycin and 0.5 μ M vemurafenib). Dotted line represents the IC_{90} value.

treatment with BRAF inhibitors and can be given concomitantly. Based on the observation of the Valpione *et al*, the tolerability of the combined treatment is of great importance, as was observed in the described case.¹² The possibility of the enhanced effectiveness further supports the approach to use ECT concomitantly during the treatment with BRAF inhibitors; however, further clinical studies with larger number of patients are needed to fully support the fact that the combined treatment does not cause additional undesired side effects. If the interaction of the treatments will be observed also *in vivo*, this may lead also to reduction of BLM dosage in ECT.

The interaction of BRAF inhibitors with radiotherapy has also been demonstrated, both on tumor and normal tissue. *In vitro* study demonstrated radiosensitization of BRAF mutated melanoma cells with BRAF inhibitor PLX-4032 by clonogenic and invasion assay and was associated with enhancement of G1 cell cycle arrest.¹⁵ Furthermore, the combination of BRAF inhibitor and irradiation was proven to be effective also in high-grade gliomas, harboring BRAF V600E mutation. Radiosensitization was observed by PLX-4720 BRAF inhibitor *in vitro*, whereas in BRAF non-mutated glioma cells the radiosensitizing effect was not observed.¹⁶ The clinical studies, however, demonstrated the radiosensitization of normal tissue as well. There is still not consensus whether the normal tissue damage is acceptable¹⁷, or that they are so severe that this requires profound investigations in the future.¹⁸

Eventually ECT will most probably find its place in combination with systemic treatments, with targeted therapies as well as with recently emerging immune checkpoint inhibitors. ECT was recently evaluated also combined with the immunotherapeutic approaches, immune checkpoint inhibitors. The first clinical study of Mozillo *et al*. reported on safety of the combined ipilimumab and ECT with good therapeutic responsiveness.¹⁹ The second report also demonstrated effectiveness and safety of a sequential treatment with ECT plus ipilimumab, which induced a durable complete response of multiple cutaneous metastases with vitiligo-like lesions indicating on involvement of immune response.²⁰ Obviously ECT is progressing into concomitant treatment with melanoma targeted therapies and immunotherapies, therefore its safety and effectiveness needs to be established. Specifically, due to induction of the immunogenic cell death induced in tumors²¹, ECT of tumors may serve as *in situ* vaccination that could be boosted by con-

comitant immunotherapeutic approach.²² The consequences may be both, the potentiated local response and also increased side effects.

In conclusion, the effectiveness of ECT in BRAF mutated cells implies on clinical applicability of ECT in BRAF mutated melanoma tumors. Furthermore, its effectiveness also during the treatment with BRAF inhibitors was demonstrated, with synergistic effectiveness. These results are encouraging, but need to be extended to more cell lines, and *in vivo* studies on experimental tumors, evaluating both the tumor and normal tissue response. The study should also be extended to patient-derived melanoma cell lines and also on clones which develop resistance to the therapy with BRAF inhibitors to verify if ECT maintains its effectiveness on the resistant clones. Such studies will then predict the tumor response, and possible side effects.

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Elektrokemoterapija z bleomicinom je učinkovita na BRAF mutiranih melanomskih celicah in ima potencirano delovanje z inhibitorji BRAF

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Izhodišča. Namen raziskave je bil ugotoviti učinkovitost elektrokemoterapije (ECT) med zdravljenjem bolnikov z melanomom z inhibitorji BRAF. Testirali smo učinkovitost ECT na BRAF mutiranih in nemutiranih melanomskih celicah *in vitro* in v kombinaciji z inhibitorjem BRAF.

Materiali in metode. ECT z bleomicinom smo izvedli na dveh humanih melanomskih celičnih linijah, eni z BRAF V600E mutacijo (SK-MEL-28) in eni brez BRAF mutacije (CHL-1). Učinkovitost ECT in ECT v kombinaciji z inhibitorjem BRAF vemurafenibom smo ovrednotili s preživetjem celic, ki smo ga določevali s testom klonogenosti.

Rezultati. Preživetje melanomskih celic z BRAF V600E mutacijo je bilo po ECT manjše kot pri celicah brez mutacije, kar kaže na to, da je ECT učinkovita ne glede na mutacijski status melanomskih celic. Dokazali smo tudi sinergistično delovanje ECT z bleomicinom in vemurafeniba v BRAF V600E mutiranih melanomskih celicah.

Zaključki. Učinkovitost ECT v BRAF mutiranih melanomskih celicah in njena potencirana učinkovitost v kombinaciji z vemurafenibom *in vitro* kaže na klinično uporabnost ECT pri bolnikih z melanomom z mutacijo BRAF in/ali med zdravljenjem z inhibitorji BRAF.

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Odkritje 'klik' 1,2,3-triazolijevih soli kot potencialnih antitumorskih učinkovin

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Izhodišča. Da bi povečali učinkovitost zdravljenja raka, smo pripravili in testirali nove potencialno antitumorsko aktivne spojine. Poročamo o testiranju novega tipa spojin, 1-(2-pikolil)-, 4-(2-pikolil)-, 1-(2-piridil)- in 4-(2-piridil)-3-metil-1,2,3-triazolijevih soli ter njihovih 1,2,3-triazolskih prekurzorjev.

Metode. Citotoksičnost novih spojin smo določali s spektrometričnim testom 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolijev bromid (MTT) na več tumorskih in eni normalni celični liniji. Učinek vezave izbranih spojin na dvojno vijačnico DNA (ds DNA) smo ugotavljali s testiranjem vpliva na termično stabilnost DNA telečjega timusa, medtem ko smo vpliv na celični ciklus določevali s pretočno citometrično analizo. Tvorbo reaktivnih kisikovih spojin (ROS) smo določevali z dodatkom specifičnega substrata, 5-(in-6)-klorometil-2',7'-diklorodihidrofluorescein diacetata, acetil estra (CM-H₂DCFDA).

Rezultati. Osnovni triazoli na splošno niso aktivni, medtem ko so triazolijeve soli zelo citotoksične na celice HeLa. Triazolijeve soli so pokazale veliko specifično citotoksičnost na različne tumorske celice. Ena od spojin, 3-metil-4-(4-metoksifenil)-1-(2-pikolil)-1H-1,2,3-triazolijev heksafluorofosfat(V) (2b), je bila bistveno bolj citotoksična na tumorske kot pa na normalne celice, z visokim terapevtskim indeksom 7,69 za celice pljučnega raka H460. Ta spojina je bila podobno aktivna na osnovne celice raka grla HEp-2 in na njihovo na zdravila odporno podlinijo 7T, kar kaže na njen potencial v zdravljenju na zdravila odpornih rakov. Spojina 2b je ustavila celice v G1 fazi celičnega cikla. Ni vezala ds DNA, temveč je inducirala ROS v obdelovanih celicah, kar je sprožilo celično smrt.

Zaključki. Naši rezultati nakazujejo, da je smiselno nadaljevati raziskave 'klik' triazolijevih soli kot antitumorskih učinkovin.