UDC 577:616 https://doi.org/10.15407/biotech17.02.064

CYTOSTATIC EFFECT OF DOXORUBICIN-HYDROCHLORIDE WITH CRM197, AN INHIBITOR OF HB-EGF, IN SQUAMOUS-CELL CARCINOMA

I.I. RADEVYCH[1, 2], *A.A. SIROMOLOT*[2, 3], *D.V. KOLYBO*[2]

[1]Educational Scientific Institute of High Technologies of Taras Shevchenko National University of Kyiv, Ukraine
[2]Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine, Kyiv
[3]ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv, Ukraine

E-mail: ***radevychina@knu.ua***

Received 2024/03/18
Revised 2024/04/04
Accepted 2024/04/30

Cancer is one of the greatest threats to public health, its lethality comparable only to that of cardiovascular diseases, with the approach to its prevention and therapy evolving from population-based and epidemiological studies to targeted delivery and immunological approaches.

Recent studies have shown that cross reacting material (CRM197), a nontoxic variant of diphtheria toxin may play an important role in treating cancers with poor prognoses by inhibiting heparin-binding EGF-like growth factor (HB-EGF) [1, 2]. Doxorubicin-hydrochloride (DOX) is an antineoplastic prescription medicine approved by the U.S. Food and Drug Administration for the treatment of certain types of cancer, including ovarian cancer and multiple myeloma [3]. Considering that CRM197 is a known carrier in targeted delivery, CRM197-DOX complexes might be a step towards targeted therapy and reduced overall toxicity.

Aim. To explore the potential to inhibit the growth of tumour cells which express EGF receptors, in this study, we evaluated the usage of CRM197-DOX complexes in squamous carcinoma cell line *A431* and compared it with the effect on other immortalised cell lines such as *3T3* fibroblasts and kidney epithelial *Vero* cells.

Methods. *Cell lines.* All cell lines were cultivated in a humidified 37 °C incubator with 5% CO_2, using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics.

Protein extraction and purification. For recombinant CRM197 and HB-EGF protein expression, *E.coli,* containing pET28(a)-CRM197 and pET32(a)-HB-EGF plasmids respectively, were grown in the lysogeny broth with the edition of an antibiotic (Kanamycin or Ampicillin) for ~2 h at 37 °C. Later, each culture was induced with IPTG and was further grown for ~3 h at 30 °C. Both cultures were centrifuged, pellets resuspended in separate tubes with the buffer containing 6 M urea and lysed by ultrasound homogenisation. Homogenized cell mass was centrifuged and the supernatants containing CRM197 and HB-EGF were retained and used for the purification of respective proteins. PolyHis labelled CRM197 and HB-EGF were purified by Ni^{2+}-NTA affinity chromatography and eluate fractions were dialysed in phosphate-buffered saline (PBS) [4]. Aliquots of proteins with sodium dodecyl sulfate (SDS) loading buffer were used for 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G-250. The expression of CRM197 and HB-EGF was confirmed by comparing bands appearing on the gel at ~58 kDa and ~10 kDa respectively.

Complex loading. 5 μM of doxorubicin was dissolved in 1 ml of 20 mM Tris-HCl buffer. The DOX solution was added to the 0.5 μg of protein. Then, the heterogenous solution was mixed for 12 h at 4 °C with further dialysis in PBS (for loaded CRM197-DOX).

MTT assay. Cells were cultivated in 96-well flat-bottomed plates in DMEM supplemented with 10% FBS and antibiotics for 24 h, then washed twice with PBS, and a fresh FBS-free culture medium

was added. Control substances were added at the following concentrations: DOX — 5 μM, HB-EGF — 0.5 μg/ml, CRM197 — 0.5 μg/ml and 1 μg/ml, CRM197 + DOX — 0.5 μg/ml of protein and 5 μM of DOX, CRM197-DOX (loaded) — 0.5 μg/ml (according to protein). After incubation for 48 h in a humidified 37 °C incubator with 5% CO_2, the optical density of the samples was measured using a multi-well spectrophotometer at 570 nm [5].

Statistics. Shapiro-Wilk test, one-way ANOVA and the Tukey–Kramer procedure were performed using Statistics Kingdom and showed significant effects for pairwise comparisons of control to test groups ($P < 0.05$).

Results and Discussion. Analytic expression with further running SDS-PAGE (Fig. 1) indicated that CRM197 is extracted in an insoluble form; purified and eluated CRM197 (~0.1 mg/ml) in the fraction C was later used in the experiment (Fig. 2).

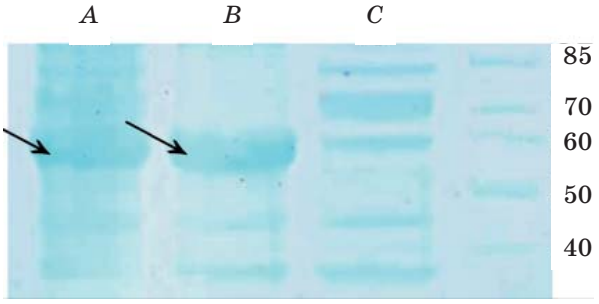

Fig. 1. **Electrophoresis assay of CRM197 analytic expression**
Lysate (*A*) and insoluble fraction (*B*) show presence of CRM197, while soluble fraction (*C*) does not

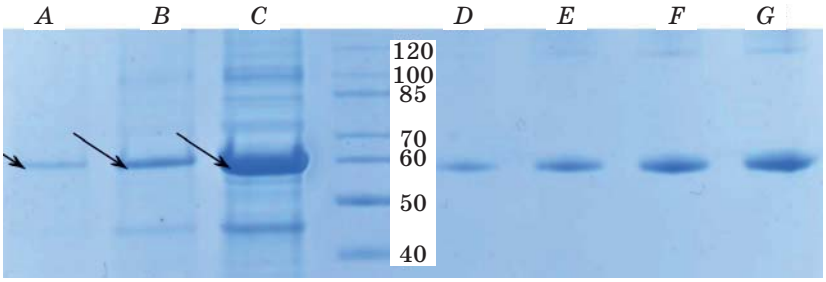

Fig. 2. **Results of SDS-PAGE of CRM197**
Different chromatography fractions showed different concentrations of CRM197 (*A*–*C*), bovine serum albumin (BSA) at given concentrations of 0.025 mg/ml (*D*), 0.05 mg/ml (*E*), 0.075 mg/ml (*F*), 0.1 mg/ml (*G*) was used to define CRM197 concentration

In the *A431* cell line (Fig. 3, *A*), treatment with CRM197 inhibited cell growth by 11% (0.5 μg/ml) and 25% (1 μg/ml). CRM197 + DOX showed results similar to those of CRM197 at 1 μg/ml concentration and of 5 μM DOX. CRM197-DOX loaded complex inhibited cell growth by 17%. It is worth noting that the excess of unbound DOX was removed by dialysis, therefore the main cytostatic effect is caused by the loaded CRM197-DOX, adding to the hypothesis that these complexes may further be used for targeted treatment of tumour cells.

The *3T3* cell line (Fig. 3, *B*) showed little to no reaction to CRM197 treatment but was excessively responding to treatment with doxorubicin-hydrochloride. CRM197 + DOX and the loaded CRM197-DOX showed results of 43% and 19% respectively.

The Vero cell line (Fig. 3, *C*) reacted to CRM197 at 0.5 μg/ml with a growth inhibition by 26%, and both 1 μg/ml concentration and CRM197 + DOX showed a result of ~37%. The loaded complex showed efficiency of 31%. All CRM197 treatments were significantly more effective than DOX.

Conclusions. CRM197-DOX complexes show evident inhibition of epidermoid carcinoma cell growth and can be used as treatment against epithelial tumours, especially those overexpressing the proHB-EGF and its receptors, EGFR1 and EGFR4, with CRM197 as a promising carrier for targeted drug delivery. Since DOX-loaded CRM197 complex shows lesser cytotoxicity than free DOX, we concluded that a certain amount of the antibiotic is lost in the process of loading, but DOX-loaded CRM197 complex still inhibits cell proliferation more strongly than free CRM197. However, there

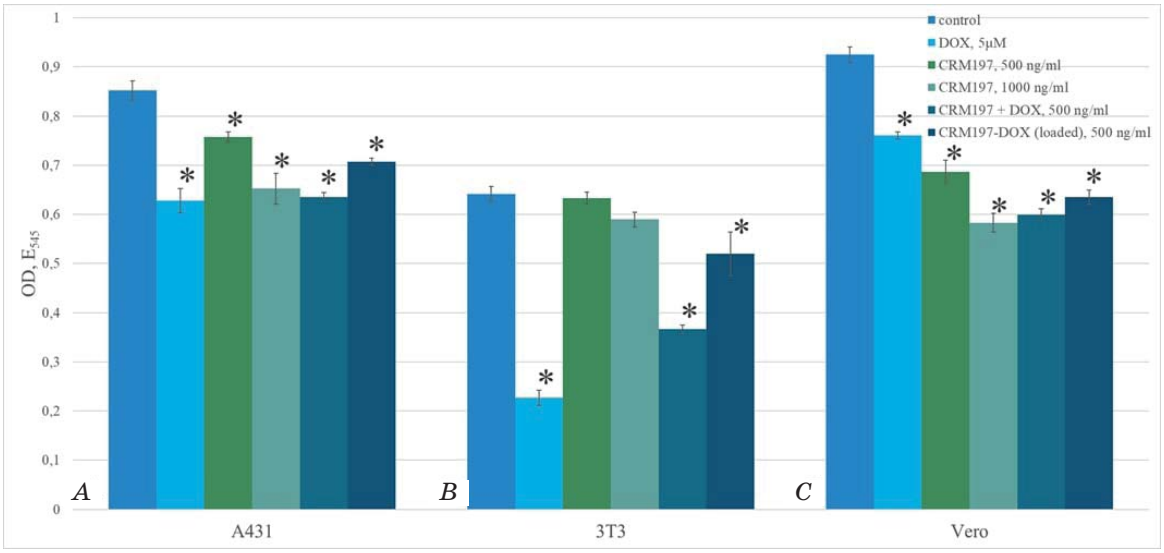

Fig. 3. **Cell viability assay results for cell line A431 (*A*), cell line 3T3 (*B*), cell line Vero (*C*)**
$*P < 0.05$

is still a need for more efficient loading techniques and to fill the gap of DOX to CRM197 loading process mechanisms.

Key words: CRM197, doxorubicin, epidermoid carcinoma, targeted treatment

Authors' contribution. I.I. Radevych conducted the experiment, protein expression and purification, performed data analysis, and wrote the manuscript. A.A. Siromolot worked with the cell culture and edited the manuscript. D.V. Kolybo suggested the study and reviewed the manuscript.

Funding source. Budget research topic of Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine "Study of receptors involved in the regulation of the organism's immunobiological functions" (No. S/r 0119U002511, 2023).

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