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HB-EGF AS A TARGETED CARRIER FOR DRUG DELIVERY IN SOME CANCERS

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Cell-penetrating peptides (CPPs) facilitate efficient biomolecule delivery with low immunogenicity and cytotoxicity, making them ideal for *in vivo* drug delivery. Heparin-binding epidermal growth factor-like growth factor (HB-EGF), a ligand of the epidermal growth factor receptor (EGFR), is overexpressed in tumors and can promote angiogenesis. Doxorubicin (DOX), a chemotherapeutic, treats various cancers but has limited use with HB-EGF as a carrier.

Aim. In this study we focused on the HB-EGF's potential in enhancing DOX delivery and antitumor effects.

Methods. Recombinant sHB-EGF was expressed in *E. coli*, purified by IMAC, and loaded with DOX with further dialysis of the complex from unbound antibiotics. Binding to cell surfaces (*A431, 3T3, Vero*), as well as ROS production using DCFH-DA, were analyzed by flow cytometry. Cell viability was assessed with MTT assays after 48 h.

Results. sHB-EGF fluorescent derivatives effectively bound to *A431* cells, enhancing DOX delivery to squamous-cell carcinoma and significantly reducing cell viability.

Conclusions. HB-EGF efficiently delivers DOX into cells, suggesting its potential as a targeted drug carrier for EGFR/ErbB-1 overexpressed cancers.

Key words: targeted drug delivery, sHB-EGF, doxorubicin, EGFR, cancer therapy.

Cell-penetrating peptides (CPPs) have been shown to be promising transport systems, capable of efficiently delivering biomacromolecules into cells [1]. Human-derived CPPs, such as heparin-binding epidermal growth factor-like growth factor (HB-EGF), offer significant advantages due to their low immunogenicity and cytotoxicity, making them ideal candidates for in vivo drug delivery. HB-EGF, a ligand for the epidermal growth factor receptor (EGFR), not only facilitates intracellular drug transport but also selectively targets cancer cells, as EGFR/ErbB-1 is frequently overexpressed in various malignancies [2].

Previous research has focused on developing drug-loading platforms for doxorubicin (DOX), a widely used anthracycline-based chemotherapeutic agent effective against various cancers, including those of the soft tissue, bone, breast, ovary, bladder, and thyroid [3]. While its precise mechanisms remain unclear, DOX is known to intercalate into DNA, inhibit topoisomerases (which leads to DNA breaks and prevents its reparation by ligation), disrupt mitochondrial function, and increase iron-mediated formation of free radicals (reactive oxygen species (ROS)), leading to diverse cell death pathways depending on dose and cellular context [4]. However, its clinical

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use is compromised by nonspecific distribution, resulting in severe side effects such as cardiotoxicity [5]. Therefore, an advanced drug delivery system is essential to enhance DOX's therapeutic efficacy while minimizing off-target toxicity.

The potential of using HB-EGF as a carrier for anticancer treatment has remained largely unexplored despite its favorable characteristics. This study investigates the ability of HB-EGF to serve as a selective drug carrier for DOX, aiming to improve its intracellular accumulation, enhance anticancer efficacy, and minimize systemic toxicity.

Materials and Methods

Protein extraction and purification. E. coli Rosetta (DE3) cells containing the p*ET*28a-HB-EGF construct were grown in LB medium with kanamycin for ~2 h at 37 °C. The culture was then induced with isopropyl β-d-1-thiogalactopyranoside (IPTG) and further grown for ~3–4 h at 30 °C and active aeration. Cells were centrifuged, and the precipitate was resuspended in Wash buffer (50 mM Na_2HPO_4, 0.3 M NaCl, pH 8.0) containing 10 mM imidazole, lysozyme (0.1 mg/mL) and with the addition of protease inhibitor cocktail, then lysed by ultrasound homogenization. The homogenized cell mass was centrifuged, and the supernatant containing HB-EGF was retained for protein purification. The HB-EGF was purified by metal-affinity chromatography (IMAC) with Co^{2+}-NTA agarose. Recombinant 6His-HB-EGF was eluted with a Wash buffer (50 mM Na_2HPO_4, 0.3 M NaCl, pH 8.0) that contained 250 mM imidazole. The purity of the recombinant proteins was confirmed via 10% SDS–PAGE under denaturing conditions, and protein concentration was quantified using densitometry with the Fiji image processing tool.

Complex loading. In order to load HB-EGF with DOX, a 5 μM DOX solution was mixed with each 500 ng of recombinant HB-EGF, and the solution was incubated for 12 hours at 4 °C with gentle stirring. Following incubation, the mixture was dialyzed against PBS (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH 7.4) to remove unbound DOX.

Cell lines. Cell lines A431, 3T3, and Vero were obtained from the Kavetsky Institute of Experimental Pathology, Oncology, and Radiobiology (Kyiv, Ukraine). All cell lines were cultured in RPMI-1640 medium supplemented with L-glutamine, 10% fetal calf serum, streptomycin (100 mg/L), penicillin (10.000 U), and amphotericin B (250 μg/L) in a 5% CO_2 atmosphere.

Cell binding assay. A431 cells were stained with mCherry and mCherry-HB-EGF to evaluate the functional activity of sHB-EGF. Fluorescence intensity was measured using a DxFLEX Flow Cytometer, and cell death was assessed via Annexin V-EGFP and propidium iodide (PI) staining.

Measurement of ROS generation. Intracellular reactive oxygen species (ROS) generation was measured using 5-(-6)carboxy-2,7-dichlorofluorescein diacetate (DCFH-DA) staining. After 24 hours of treatment with sHB-EGF-DOX complexes and DOX, the A431 and Vero cells were incubated with DCFH-DA (10 μM in PBS) for 30 min at room temperature. Fluorescence was captured using a DxFLEX Flow Cytometer with further data processing using Kaluza Analysis Software (Beckman Coulter, USA).

MTT assay. Cell viability was assessed using the MTT reagent. Cells (100 μL of suspension containing 25,000 cells) were placed into 96-well plates and incubated for 24 hours. After two washes with PBS, fresh serum-free medium was added with control substances at the following concentrations: DOX — 5 μM, sHB-EGF — 500 ng/ml, sHB-EGF + DOX (mix) — 500 ng/ml of protein and 5 μM of DOX, sHB-EGF -DOX (loaded complexes) — 500 ng/ml (according to protein), and the cells were further incubated for 48 hours at 37 °C. MTT reagent (0.5 mg/mL) was then added to treated cells, and the plates were incubated for 4 hours at 37 °C. The results were recorded by measuring absorbance at 570 nm using a multi-well spectrophotometer MULTISKAN FC.

Statistical analysis. The statistical significance of differences between samples was determined using the Student's T-test, with $P < 0.05$ considered statistically significant.

Results and Discussion

The purification of recombinant proteins human sHB-EGF and mCherry-sHB-EGF was confirmed via affinity chromatography and polyacrylamide gel electrophoresis. The molecular masses of the proteins corresponded to the theoretically predicted values (Fig. 1).

Flow cytometric analysis of A431 cells stained with mCherry and mCherry-HB-EGF revealed a noticeable rightward shift in the fluorescence peak, indicating specific binding of the labeled HB-EGF ligand to EGFR on the cell surface. This shift represented a 60%

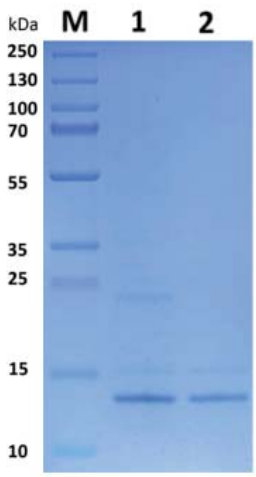

Fig. 1. **Results of SDS-PAGE of recombinant human 6His-sHB-EGF.** ***1, 2*** different fractions of 6His-HB-EGF (0.5–1 mL and 1–1.5 mL collected flow samples on column respectively)
M — protein molecular weight marker (PageRuler™ Prestained Protein Ladder, 10 to 250 kDa, Thermofisher)

change in fluorescence compared to control cells (Fig. 2).

The cytotoxicity and anti-proliferative activities of DOX at different concentrations (0.1 μM, 0.5 μM, 5 μM, 10 μM, 25 μM, 50 μM) were assessed using MTT assay in various cell lines (A431, 3T3, Vero). DOX concentrations of 5 μM and higher significantly reduced cell viability compared to controls (Fig. 3).

The impact of DOX-loaded HB-EGF on cell viability was further evaluated in A431, 3T3, and Vero cells at various concentrations of HB-EGF and DOX-loaded HB-EGF. Both DOX-loaded HB-EGF and HB-EGF combined with DOX significantly decreased cell viability compared to control wells and HB-EGF alone (Fig. 4). No statistically significant differences were observed between the HB-EGF with DOX and DOX-loaded HB-EGF groups.

One of the key findings of this study is the comparable cytotoxicity observed between HB-EGF with DOX and DOX-loaded HB-EGF, suggesting that HB-EGF effectively interacts with DOX to facilitate its intracellular delivery. This supports the hypothesis that HB-EGF, as a naturally occurring ligand of EGFR, enhances DOX uptake into EGFR-expressing cells. The absence of significant differences between these two treatment groups may indicate that HB-EGF does not require a specific conjugation method to function as an efficient DOX transporter.

A notable advantage of using HB-EGF as a drug carrier is its potential to minimize systemic toxicity. Since HB-EGF selectively binds to EGFR, which is overexpressed in various cancers, this approach may reduce off-target effects and improve therapeutic outcomes. However, additional research is needed to assess the selectivity of DOX-loaded HB-EGF in *vivo* models, as well as to determine whether it can overcome common resistance mechanisms associated with DOX treatment.

Moreover, doxorubicin suppressed the proliferative potential of the growth factor in all cell lines, including cancer cells. This also indicates that the targeted delivery system was effective, as HB-EGF specifically bound to cell receptors, allowing doxorubicin to exert its proliferation-inhibiting effects.

We established that DOX induces ROS generation in DOX-loaded HB-EGF complexes

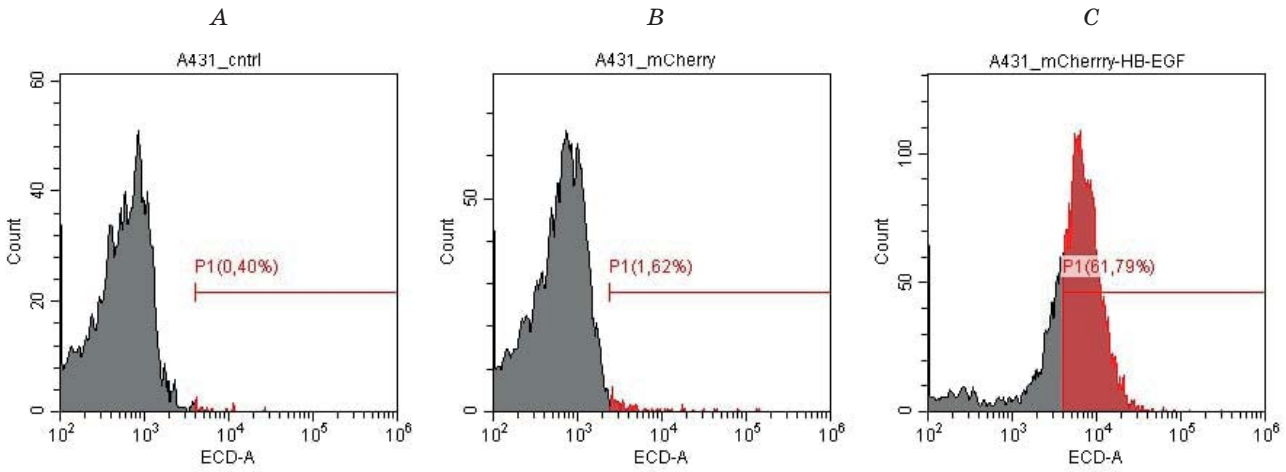

Fig. 2. **Fluorescence intensity on the ECD-A channel in control unstained (*A*), mCherry-stained (*B*), and mCherry-HB-EGF-stained (*C*) A431 cell samples**

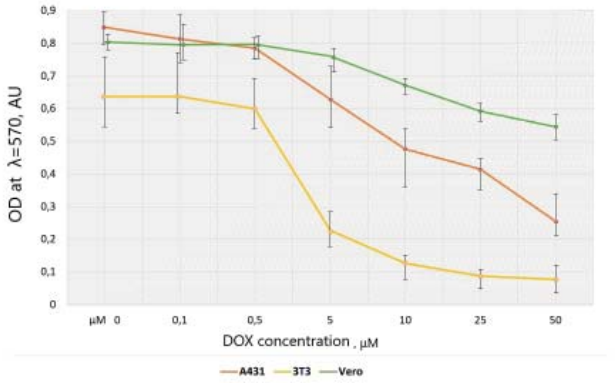

Fig. 3. **MTT assay results for A431, 3T3, and Vero cell lines exposed to varying DOX concentrations**

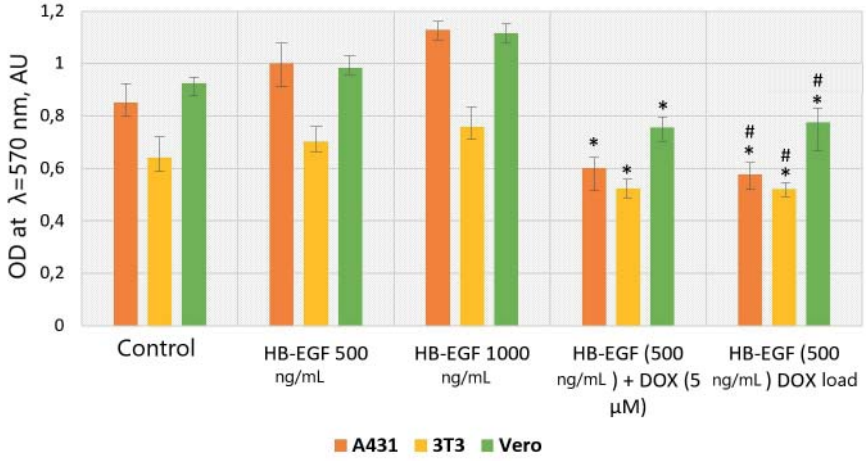

Fig. 4. **MTT assay results for A431, 3T3, and Vero cells treated with HB-EGF (500 ng/mL and 1000 ng/mL), HB-EGF with DOX (5 μM), and DOX-loaded HB-EGF**
* $P < 0.05$ compared to control, $P < 0.05$ compared to HB-EGF (500 ng/mL), $P < 0.05$ compared to HB-EGF (1000 ng/mL).
\# $P < 0.05$ compared to HB-EGF (500 ng/mL) + DOX.

in cancer cell treatment. Figure 5 depicts that the cellular oxidative stress increased significantly in treated cells compared to untreated cells. Production of ROS by HB-EGF-DOX complexes is relatively lower compared to that of pure DOX. However, the utilization of sHB-EGF as a ligand to deliver DOX to sensitive cells represents a highly effective strategy. This targeted delivery approach enhances the therapeutic efficacy of DOX while potentially reducing off-target effects and minimizing systemic toxicity. By directing the chemotherapeutic agent specifically to cancer cells, the protein-mediated targeting mechanism offers a promising avenue for improving cancer treatment outcomes. Oxidative damage is often leveraged in cancer therapy to induce apoptosis in cancer cells. DOX is well-known for its ability to generate ROS and induce apoptosis in cancer cells. When combined with sHB-EGF, which targets EGFR, the effectiveness of DOX may be enhanced, leading to increased ROS generation and subsequent cell death in targeted cells.

Despite the promising findings, several limitations should be addressed in future studies. The precise mechanism of DOX loading onto HB-EGF remains to be elucidated, and optimization of binding efficiency could enhance the therapeutic potential of this approach. In some studies, DOX has been conjugated to an EGFR-binding peptide via an ester bond at position 14, using a glutarate spacer for targeted drug delivery [6]. Additionally, the stability of DOX-HB-EGF complexes in physiological conditions needs to be further characterized to ensure adequate drug release upon cellular uptake.

Overall, our findings suggest that HB-EGF is a viable candidate for targeted DOX delivery, with potential applications for cancer

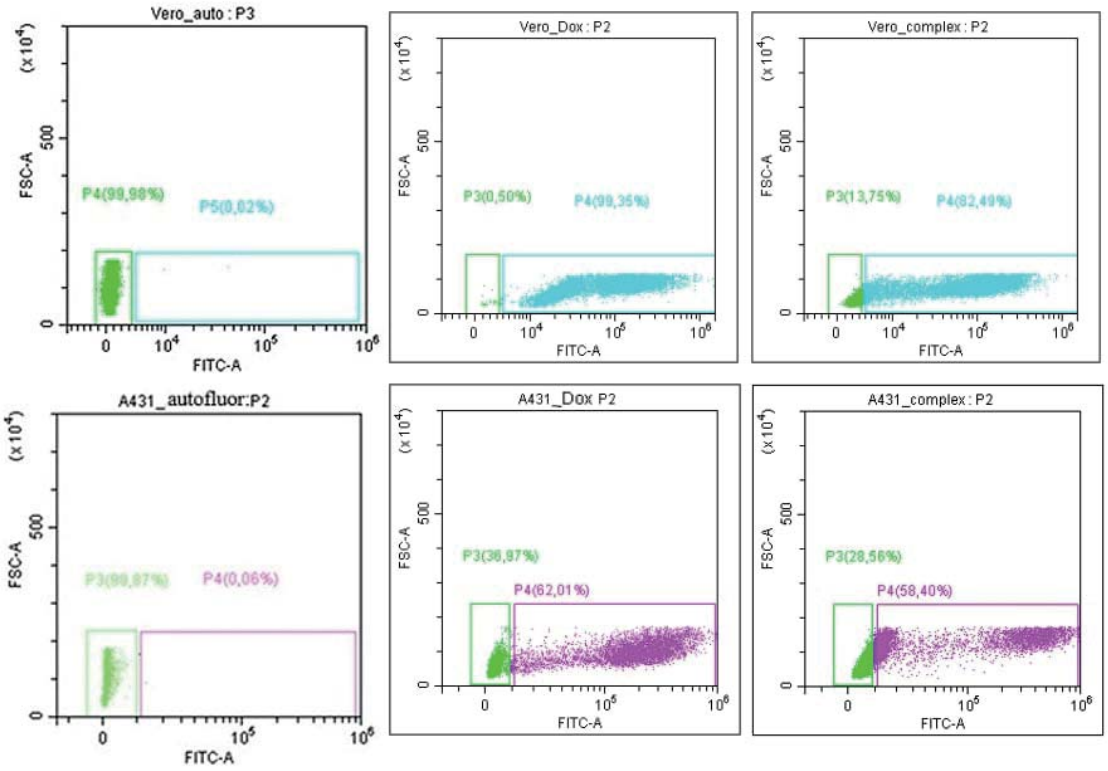

Fig. 5. **Flow cytometry analysis representing the percentage of cells after DOX-induced elevation of intracellular ROS level in A431 and Vero cells treated with DOX-loaded HB-EGF complexes**

therapy. Further studies focusing on *in vivo* efficacy, long-term stability, and mechanistic insights will be essential to validate its clinical utility.

Conclusions

This study demonstrates that DOX-loaded HB-EGF significantly inhibits cell growth and viability across multiple cell lines. Both DOX-loaded HB-EGF and HB-EGF combined with DOX exhibited comparable effects in reducing cell viability compared to controls. Notably, the targeted delivery of DOX via sHB-EGF enhances therapeutic efficacy while potentially minimizing off-target effects and systemic toxicity. These findings highlight the potential of HB-EGF as an effective carrier for targeted DOX delivery in cancer therapy. Further investigation is needed to explore the enhancement of DOX's antitumor effects through HB-EGF and to understand the mechanisms of DOX-protein loading better.

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Authors' contribution

Conceptualization, A.S., and D.K.; methodology, data collection, software, writing — original draft preparation I.G. and A.S.; writing — review and editing, D.K.; supervision, A.S., and D.K. All authors have read and agreed to the published version of the paper.

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HB-EGF ЯК НОСІЙ ДЛЯ ТАРГЕТНОЇ ДОСТАВКИ ЛІКАРСЬКИХ ЗАСОБІВ ПРИ ДЕЯКИХ ВИДАХ РАКУ

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Пептиди, що проникають у клітини (CPP), забезпечують ефективну доставку біомолекул із низькою імуногенністю та цитотоксичністю, що робить їх придатними для *in vivo* транспорту лікарських засобів. Гепарин-зв'язувальний фактор росту, подібний до епідермального ростового фактору (HB-EGF), ліганд рецептора епідермального фактора росту (EGFR), надекспресується в пухлинах і здатен стимулювати ангіогенез. Доксорубіцин (DOX), протипухлинний препарат широкого спектра дії, використовується в терапії різних онкопатологій, проте його застосування в комбінації з HB-EGF як носієм залишається малодослідженим.

Мета. Дослідити потенціал HB-EGF для доставки DOX і посилення його протипухлинної дії.

Методи. Рекомбінантний протеїн sHB-EGF експресували в *E. coli*, очищали методом IMAC, інкубували з DOX, після чого проводили діаліз для видалення незв'язаного препарату. Здатність до зв'язування з клітинними рецепторами (*A431*, *3T3*, *Vero*) та утворення активних форм кисню (DCFH-DA) аналізували методом протокової цитофлуориметрії. Життєздатність клітин оцінювали за допомогою MTT-тесту через 48 год.

Результати. Флуоресцентно мічені похідні sHB-EGF ефективно зв'язувалися з клітинами *A431*, сприяючи доставці DOX до клітин, що походять з плоскоклітинної карциноми, та достовірному зниженню життєздатності клітин.

Висновки. HB-EGF є ефективним носієм для доставки доксорубіцину в клітини, що свідчить про його потенціал як платформи для таргетної терапії пухлин з надекспресією EGFR/ErbB-1.

Ключові слова: таргетна доставка ліків, sHB-EGF, доксорубіцин, EGFR, протипухлинна терапія.