



HSP70 Inhibition Alters Extracellular Vesicles Activity *In Vitro*

Mohamad Zulfikrie Mohd Zulkifli¹, Siti Fathiah Masre¹, and Nadiah Abu^{*2}

¹Department of Biomedical Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia.

²UKM Medical Molecular Biology Institute (UMBI), Universiti Kebangsaan Malaysia, 56000 Kuala Lumpur, Malaysia.

KEYWORDS

exosome
cancer
heat shock protein

ABSTRACT

Heat shock protein 70 (HSP70) is a stress-induced molecular chaperone that is frequently overexpressed in many cancers, including colorectal cancer (CRC). In addition to its intracellular functions, HSP70 has been detected in extracellular vesicles (EVs) as well. EVs are nanovesicles that are released into the extracellular space and are known to promote cancer cell proliferation and migration. However, the role of HSP70 in influencing EV function remains unclear. This study examined the effects of HSP70 inhibition on EVs derived from the HT-29 colorectal cancer cell line. The HSP70 inhibitor VER-155008 was used to suppress HSP70 activity. Flow cytometric analysis confirmed that the isolated EVs expressed the characteristic surface markers CD63, CD81, and CD9. Wound healing assays showed a marked reduction in cell migration following VER-155008-EV treatment at both 24 and 48 hours. These findings suggest that inhibition of HSP70 alters the properties of EVs and may influence their ability to support tumor cell migration. This study provides insight into the potential role of HSP70 in modulating EV function in CRC.

ARTICLE HISTORY

Received 31 Oct 2025
Received in revised form
31 Aug 2026
Accepted 31 Aug 2026
Available online 19 Sep 2026

© 2026 The Authors. Published by Penteract Technology.

This is an open access article under the CC BY-NC 4.0 license (<https://creativecommons.org/licenses/by-nc/4.0/>).

1. INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer globally and the incidence of CRC is rising year after year [1]. In Malaysia, CRC is the second most prevalent cancer, accounting for 13.5% of all newly diagnosed cases from 2012 to 2016 [2]. There are various risk factors that are associated with CRC including obesity, diet, alcohol and tobacco [1]. Heat shock protein 70 (HSP70) is a molecular chaperone expressed in response to cellular stress. It stabilizes proteins by binding to them, preventing denaturation or aggregation until normal conditions are restored [3]. HSP70 plays several key roles in normal cellular processes, such as facilitating the folding of newly synthesized proteins, transporting proteins and vesicles, assembling and disassembling complexes, and degrading damaged or unwanted proteins [3]. This chaperone is critical for maintaining protein homeostasis under both stress and normal conditions [4]. While in homeostasis, inducible HSP70 levels are modest; however, damage significantly increases its expression. Consequently, it has been shown that HSP70 is a sign of cellular stress and can be translocated to the extracellular space [5].

Structurally, HSP70 contains an N-terminal nucleotide-binding domain (NBD) (~40 kDa) and a C-terminal substrate-binding domain (SBD) (~25 kDa), connected by a short linker [6]. High expression of HSP70 is associated with poor prognosis in several cancers, including breast, endometrial, oral, colorectal, and prostate cancers, as well as certain leukemias [7]. HSP70 interacts with components of both caspase-dependent and independent apoptotic pathways, and its overexpression is believed to give cancer cells a survival advantage [8]. Extracellular HSP70 has been long thought to be a promising biomarker for various diseases including cancers and other haematological malignancies [9].

Extracellular vesicles (EVs) are membrane-bound vesicles that transport bioactive molecules between cells. There are several types of EVs depending on the size and biogenesis, including apoptotic bodies, microvesicles, and exosomes [10]. EVs cargo can alter the fate of recipient cells, both locally and remotely, and they often serve as pathological biomarkers [10]. HSP70 positive EVs have been shown to be involved in various cellular processes. For instance, in an earlier study by Gastpar et al., in 2005 has shown that EVs containing HSP70 stimulate cytolytic activity of natural killer cells [11]. EV-

*Corresponding author:

E-mail address: Nadiah Abu <nadiah.abu@hctm.ukm.edu.my>.

<https://doi.org/10.56532/mjsat.v6i3.645>

2785-8901/ © 2026 The Authors. Published by Penteract Technology.

This is an open access article under the CC BY-NC 4.0 license (<https://creativecommons.org/licenses/by-nc/4.0/>).

bound HSP70 has been shown to be upregulated in different cancers such as glioma[12] and breast cancer [13]. Inhibition of HSP70 has been a viable approach for treating cancers [14, 15]

In fact, multiple small molecule inhibitors have been developed to evaluate the efficacy of HSP70 inhibitors in different cancers [16]. Nevertheless, the effects of HSP70 inhibition on the downstream activity of the released EVs have yet to be investigated yet. Therefore, herein we report the effects of HSP70 inhibition on the functional activities of the EVs in CRC.

2. MATERIALS AND METHODS

2.1 Cell Culture

The colorectal cancer cell line, HT-29 was used in this study. This cell line was previously obtained from ATCC and was kept in UMBI's Biobank. The cells were seeded and cultured in RPMI-1640 medium (Nacalai-Tesque, Japan) supplemented with 10% EV-depleted FBS and 1% Penn-Strep (Nacalai Tesque, Japan). The cells were incubated at 37 °C with 5% CO₂. For EV isolation, 7.0×10^6 cells were cultured in the two T-175 flasks. One was untreated (UT) group meanwhile the other one was the VER-155008 treated (TVER) group. For the TVER group, treatment was given towards the cells after reaching the 70-80% confluency. 0.5 μ M VER-155008 is being added into the EV-depleted cell culture supernatant media. After 24 hours, the cell culture supernatant for both groups were then collected for EV isolation

2.2 Extracellular Vesicle (EV)-Enriched Isolation

EVs were isolated from the cell culture supernatant of the cultured cells. The EVs were subjected to precipitation and column-based isolation (ExoEasy Maxi Column) following the manufacturer's protocol (Qiagen, Germany).

2.3 BCA Measurement

The isolated EVs were subjected to quantification using the Pierce BCA Assay Kit according to the manufacturer's instructions (Thermo Fisher, USA).

2.4 VER155008 Treatment

We screened several concentrations of VER 155008 (MedChemExpress, USA) on HT-29 cells using MTT assay (Sigma, USA). In brief, 1×10^5 cells/ml of HT-29 cells were seeded in a 96 well-plate overnight. The next day, various concentrations of VER155008 were added to the wells and left to incubate for 48 and 72 hours. Following the incubation time, 5 mg/mL of MTT was added to each of the wells and were incubated for 3-4 hours. Afterwards, the crystallized mixture was dissolved with DMSO and the absorbance was read at 560nm using a microplate reader.

2.5 CCK 8 Analysis

The CCK-8 assay was done according to the manufacturer's protocol (Biosharp, China). In brief, 1×10^4 of HT-29 cells were seeded in a 96 well-plate overnight. Then, the cells were either treated with EVs isolated from the VER-treated cells. After 24 hours, 10 μ L of CCK-8 was added to each of the wells and was left to incubate for 2 hours before the absorbance of the reactions was read at 450nm using a microplate reader.

2.6 Wound Healing Analysis

At the beginning of the experiment, 4.0×10^5 HT-29 cells were cultured in each well with 500 μ L of medium with EVS-depleted FBS. After that, 300 μ L of the medium was discarded, and the surface of each well was scratched with the tip of a 200 μ L pipette. Each well was washed twice with 500 μ L of 1X PBS. Then, the cells were treated with the EVs isolated from the VER-treated cells. Pictures of the scratch was taken at 6h, 24h and 48h post-treatment.

2.7 Statistical Analysis

We used students T-test for statistical analysis and p value<0.05 was considered significant. The statistical analysis was performed using the Graphpad Prism software.

3. RESULTS

To determine the effect of VER-155008 on HT-29 cell viability, an MTT assay was performed with increasing concentrations of the compound. As shown in Figure 1A, VER-155008 reduced cell viability in a dose-dependent manner, with an estimated IC₅₀ value between 0.7 and 1.0 μ M. Based on these findings, a sub-cytotoxic concentration of 0.5 μ M was selected for subsequent experiments to minimize cell death and avoid potential interference with extracellular vesicle (EV) release. Following EV enrichment, flow cytometry analysis was performed to assess the presence of EV-associated surface markers. As shown in Figure 1B, EVs from both groups expressed the canonical tetraspanin markers CD63, CD81, and CD9, confirming successful enrichment of extracellular vesicles in our preparations.

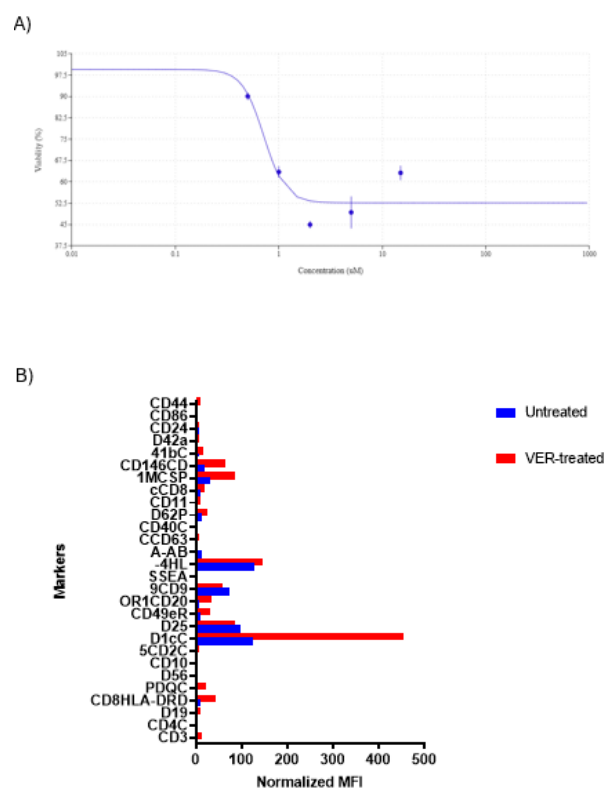


Fig. 1. A) MTT analysis after treatment of HT-29 with varying concentrations of VER-155008. B) MacsPlex EV IO

analysis of the EVs isolated from the Untreated and VER-Treated cells.

To further investigate the phenotypic effects of VER155008 in HT-29 cells we performed a wound healing analysis. As shown in Figure 2, the wound healing assay demonstrates that extracellular vesicles (EVs) derived from VER155008-treated HT-29 cells reduced cell migration compared to untreated controls and EVs from untreated cells. At 24 and 48 hours, the untreated and untreated EV groups showed increased wound closure, indicating active cell migration, whereas cells treated with VER155008-derived EVs exhibited markedly slower closure. Quantitative analysis confirmed a time-dependent increase in wound closure in all groups, but with lower migration percentages in the VER155008-treated EV group. These findings suggest that inhibition of HSP70 by VER155008 alters the functional properties of released EVs, which may diminish their pro-migratory effects on colorectal cancer cells.

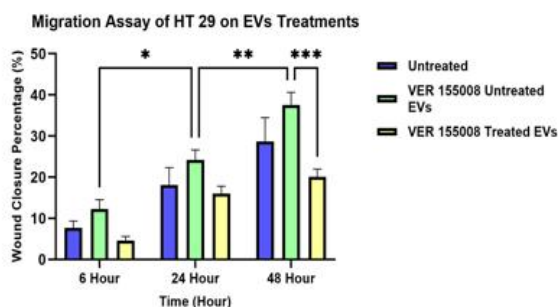
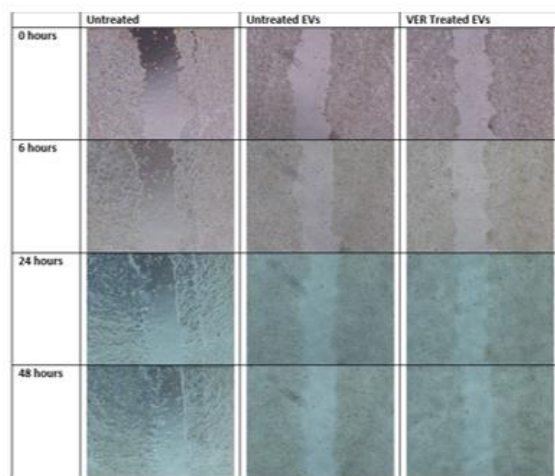


Fig. 2. Wound healing analysis of HT-29 cells after 6, 24 and 48 hours of treatment with the Untreated and VER-Treated EVs.

Additionally, we also performed a CCK-8 assay to evaluate the proliferative activity of the isolated EVs. Based on Figure 3, the proliferation assay shows the effect of extracellular vesicles (EVs) derived from untreated (UT-EVs) and VER155008-treated (T-EVs) HT-29 cells on cell viability across varying EV concentrations. Overall, both EV types maintained high cell viability, indicating no significant cytotoxic effects at the tested concentrations. However, a slight reduction in viability was observed in cells treated with higher concentrations (2–4 $\mu\text{g/mL}$) of T-EVs compared to UT-EVs, suggesting that EVs derived from VER155008-treated

cells may modestly suppress proliferation at higher concentrations.

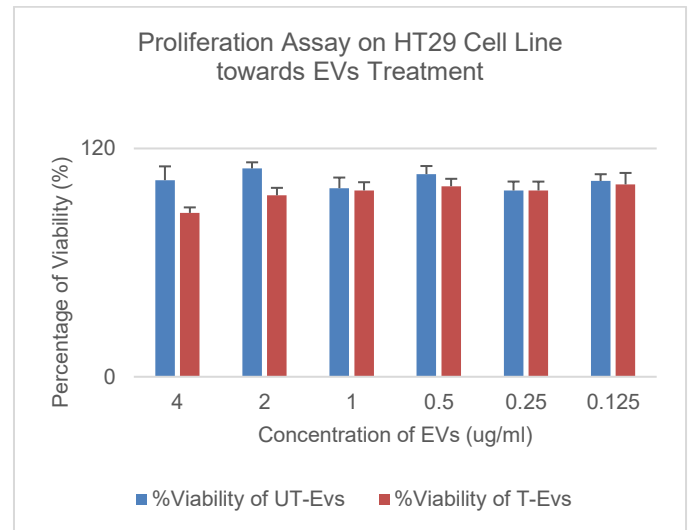


Fig. 3. CCK8-analysis of HT-29 cells treated with untreated and VER-treated EVs

4. DISCUSSION

To evaluate the effect of HSP70 inhibition on cell proliferation we conducted an MTT assay. As displayed in Figure 1A, HT-29 cells had lower cell viability as the doses of VER-155008 increased. An earlier study by Massey et al., showed that VER-155008 has a higher inhibitor potential in HCT116 cells as compared to HT-29 cells [17]. This influenced the study design by confirming HT-29 as an appropriate model for investigating sub-lethal physiological modulations because HT-29 cells tolerate low-dose treatment without undergoing immediate apoptosis. The authors report that the GI50 value for HT-29 was around 12.8 μM [17]. Based on these results, we selected 0.5 μM as the dose for the downstream functional assays as we do not want to induce cell death in HT-29 cells but rather inhibit HSP70 while still maintaining some physiological functions. We further isolated the EVs from both untreated and VER-treated cells. Both populations displayed classical EV markers such as CD63, CD81 and CD9. Nevertheless, it should be noted that after normalization the VER-treated EVs had a lower expression of these markers as compared to untreated EVs. This could probably be due to the function of HSC70/HSP70 that is heavily involved in the endosomal pathway and protein cargo selection [18]. This may be the reason why the tetraspanin markers vary between the two groups.

In our study design, it can be hypothesized that VER155008 could inhibit HSP70 that are found both on the cellular membrane, extracellular HSP70 as well as EV-bound HSP70. Multiple studies have shown that HSP70 can be found in the plasma membrane [19]. With this assumption, we postulate that the isolated EVs from the VER treated cells could also have a physiological difference. Our initial results indicated that in VER-treated cells/EVs, the migration and proliferation of HT-29 cells were slightly impeded. The slight, concentration-dependent reduction in viability observed in recipient cells treated with higher doses (2–4 $\mu\text{g/mL}$) of T-EVs indicates that inhibiting HSP70 in producer cells attenuates the baseline pro-survival and pro-proliferative

signaling capacity typically conferred by tumor EVs. It suggests that T-EVs lack critical growth-promoting cargo or possess altered molecular signals that modestly suppress cancer cell proliferation without causing overt cytotoxic destruction.

A study by Gastpar et al., has shown that HSP70-containing exosomes are able to affect the migratory and cytolytic abilities of natural killer cells [11]. This was in agreement with a different study by Lv et al., that showed hepatocellular cancer cells release EV-containing HSPs that are able to stimulate the activity of NK cells as well [20]. When it comes to cancer cells, studies have shown that extracellular HSPs can often enhance both pro-and anti-tumorigenic properties [21]. For instance, Gastpar et al., observed that HSP70 can stimulate the activity of natural killer cells [11]. The dual role of extracellular HSPs further elucidates the complexity of HSPs especially when it comes to the tumor microenvironment.

We postulate that once HSP70 is inhibited, the downstream regulation leading to EV release were also affected, thus implicating the cargoes carried within the EVs. As mentioned earlier, HSP70 is heavily involved in the protein cargo selection [18]. This could in turn affect how the EVs act when in contact with the recipient cells. For instance, in a study by Lauwers et al., showed that when HSP90 activity was inhibited especially the membrane deforming ability, the exosomes released were also affected [22].

5. CONCLUSION

Overall, our preliminary results indicate that HSP70 inhibition did have some effects on the extracellular vesicles. Nevertheless, more in depth studies are needed to fully elucidate the relationship between HSP70 and extracellular vesicles.

ACKNOWLEDGEMENT

We would like to acknowledge UKM Medical Molecular Biology Institute (UMBI) for allowing us to conduct our work using their facilities. Also, we would like to acknowledge the Loreal FWIS- JJ-2022-07 grant for providing funds to support this work. We would also like to acknowledge Mr Johannes Low Jun Wei, Ms Lim Chai Ling and Ms Siti Hasanah Yusuf for their assistance in this study. Lastly, we acknowledge the usage of an OpenAI tool, ChatGPT that was primarily used to enhance the readability of the manuscript.

REFERENCES

- [1] P. Rawla, T. Sunkara, and A. Barsouk, "Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors," *Prz Gastroenterol*, vol. 14, no. 2, pp. 89–103, 2019, doi: <https://doi.org/10.5114/pg.2018.81072>.
- [2] N. A. Muhamad et al., "Colorectal cancer survival among Malaysia population: data from the Malaysian National Cancer Registry," *Front Oncol*, vol. 13, p. 1132417, 2023, doi: <https://doi.org/10.3389/fonc.2023.1132417>.
- [3] M. P. Mayer and B. Bukau, "Hsp70 chaperones: cellular functions and molecular mechanism," *Cell Mol Life Sci*, vol. 62, no. 6, pp. 670–684, 2005, doi: <https://doi.org/10.1007/s00018-004-4464-6>.
- [4] E. Meimaridou, S. B. Gooljar, and J. P. Chapple, "From hatching to dispatching: the multiple cellular roles of the Hsp70 molecular chaperone machinery," *J Mol Endocrinol*, vol. 42, no. 1, pp. 1–9, 2009, doi: <https://doi.org/10.1677/jme-08-0116>.
- [5] V. L. Vega et al., "Hsp70 translocates into the plasma membrane after stress and is released into the extracellular environment in a membrane-associated form that activates macrophages," *J Immunol*, vol. 180, no. 6, pp. 4299–4307, 2008, doi: <https://doi.org/10.4049/jimmunol.180.6.4299>.
- [6] E. B. Bertelsen et al., "Solution conformation of wild-type E. coli Hsp70 (DnaK) chaperone complexed with ADP and substrate," *Proc Natl Acad Sci U S A*, vol. 106, no. 21, pp. 8471–8476, 2009, doi: <https://doi.org/10.1073/pnas.0903503106>.
- [7] C. G. Evans, L. Chang, and J. E. Gestwicki, "Heat shock protein 70 (hsp70) as an emerging drug target," *J Med Chem*, vol. 53, no. 12, pp. 4585–4602, 2010, doi: <https://doi.org/10.1021/jm100054f>.
- [8] Z. Albakova et al., "HSP70 Multi-Functionality in Cancer," *Cells*, vol. 9, no. 3, 2020, doi: <https://doi.org/10.3390/cells9030587>.
- [9] N. Mouawad et al., "Is It Still Possible to Think about HSP70 as a Therapeutic Target in Onco-Hematological Diseases?" *Biomolecules*, vol. 13, no. 4, 2023, doi: <https://doi.org/10.3390/biom13040604>.
- [10] M. Sheta et al., "Extracellular Vesicles: New Classification and Tumor Immunosuppression," *Biology (Basel)*, vol. 12, no. 1, 2023, doi: <https://doi.org/10.3390/biology12010110>.
- [11] R. Gastpar et al., "Heat shock protein 70 surface-positive tumor exosomes stimulate migratory and cytolytic activity of natural killer cells," *Cancer Res*, vol. 65, no. 12, pp. 5238–5247, 2005, doi: <https://doi.org/10.1158/0008-5472.Can-04-3804>.
- [12] P. Lennartz et al., "Biomarkers in Adult-Type Diffuse Gliomas: Elevated Levels of Circulating Vesicular Heat Shock Protein 70 Serve as a Biomarker in Grade 4 Glioblastoma and Increase NK Cell Frequencies in Grade 3 Glioma," *Biomedicines*, vol. 11, no. 12, 2023, doi: <https://doi.org/10.3390/biomedicines11123235>.
- [13] A. Xanthopoulos et al., "Hsp70-A Universal Biomarker for Predicting Therapeutic Failure in Human Female Cancers and a Target for CTC Isolation in Advanced Cancers," *Biomedicines*, vol. 11, no. 8, 2023, doi: <https://doi.org/10.3390/biomedicines11082276>.
- [14] S. Du et al., "Advances in the study of HSP70 inhibitors to enhance the sensitivity of tumor cells to radiotherapy," *Front Cell Dev Biol*, vol. 10, p. 942828, 2022, doi: <https://doi.org/10.3389/fcell.2022.942828>.
- [15] A. R. Goloudina, O. N. Demidov, and C. Garrido, "Inhibition of HSP70: A challenging anti-cancer strategy," *Cancer Letters*, vol. 325, no. 2, pp. 117–124, 2012, doi: <https://doi.org/10.1016/j.canlet.2012.06.003>.
- [16] X. Wang et al., "HSP27, 70 and 90, anti-apoptotic proteins, in clinical cancer therapy (Review)," *Int J Oncol*, vol. 45, no. 1, pp. 18–30, 2014, doi: <https://doi.org/10.3892/ijo.2014.2399>.
- [17] A. J. Massey et al., "A novel, small molecule inhibitor of Hsc70/Hsp70 potentiates Hsp90 inhibitor induced apoptosis in HCT116 colon carcinoma cells," *Cancer Chemotherapy and Pharmacology*, vol. 66, no. 3, pp. 535–545, 2010, doi: <https://doi.org/10.1007/s00280-009-1194-3>.
- [18] R. Sahu et al., "Microautophagy of cytosolic proteins by late endosomes," *Dev Cell*, vol. 20, no. 1, pp. 131–139, 2011, doi: <https://doi.org/10.1016/j.devcel.2010.12.003>.
- [19] A. De Maio, "Extracellular heat shock proteins, cellular export vesicles, and the Stress Observation System: A form of communication during injury, infection, and cell damage," *Cell Stress and Chaperones*, vol. 16, no. 3, pp. 235–249, 2011, doi: <https://doi.org/10.1007/s12192-010-0236-4>.
- [20] L. H. Lv et al., "Anticancer drugs cause release of exosomes with heat shock proteins from human hepatocellular carcinoma cells that elicit effective natural killer cell antitumor responses in vitro," *J Biol Chem*, vol. 287, no. 19, pp. 15874–15885, 2012, doi: <https://doi.org/10.1074/jbc.M112.340588>.
- [21] L. Secli et al., "The dark-side of the outside: how extracellular heat shock proteins promote cancer," *Cellular and Molecular Life Sciences*, vol. 78, 2021, doi: <https://doi.org/10.1007/s00018-021-03764-3>.
- [22] E. Lauwers et al., "Hsp90 Mediates Membrane Deformation and Exosome Release," *Molecular Cell*, vol. 71, no. 5, pp. 689–702.e9, 2018, doi: <https://doi.org/10.1016/j.molcel.2018.07.016>.