

TARGETING HMGB1/RAGE AXIS WITH METFORMIN: A POTENTIAL ANTI-METASTATIC STRATEGY IN LUNG ADENOCARCINOMA

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Abstract

Lung cancer remains the leading cause of cancer-related mortality world-wide, with metastasis and resistance to therapy posing significant treatment challenges. HMGB1/RAGE signalling axis plays a central role in the process of metastasis. In this study, we investigated the potential of metformin, a well-known antidiabetic agent, to inhibit HMGB1/RAGE-mediated EMT and migration in A549 lung adenocarcinoma cells.

Our findings demonstrate that recombinant HMGB1 increases RAGE expression and its localization to the cell membrane. Treatment with HMGB1 also elevates the levels of mesenchymal marker vimentin, enhancing the migratory capacity of A549 cells. All these effects were notably suppressed by co-treatment with metformin. These results suggest that metformin disrupts HMGB1/RAGE interaction and signalling, thereby inhibiting EMT and migration.

Collectively, our data support the hypothesis that metformin binds HMGB1 and prevents its interaction with RAGE, contributing to its antitumour effects. This highlights a novel anti-metastatic mechanism of metformin and underscores its potential as a complementary therapeutic strategy for targeting tumour progression in lung cancer.

Key words: HMGB1/RAGE interaction, vimentin, cell movement, lung cancer

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Introduction. Lung cancer is a major global health problem with serious social, economic, and medical implications and is the leading cause of cancer-related deaths worldwide. It often goes undetected until it is in an advanced stage, making it harder to treat. Millions are diagnosed every year, and many cases are preventable. Metastasis, drug resistance, and side effects of therapies make managing lung cancer complex. Chronic inflammation can contribute to the development and progression of many types of cancer including in the development of lung cancer [1]. High Mobility Group Box 1 (HMGB1) is a non-histone nuclear protein that plays a crucial role in chronic inflammation, acting as a damage-associated molecular pattern (DAMP) molecule which can influence various biological processes, including immune responses and cancer progression [2].

In the context of lung cancer, HMGB1 has been implicated in promoting tumour progression and metastasis, including by facilitating epithelial-mesenchymal transition (EMT) [3]. EMT is a biological process in which epithelial cells lose their characteristic features and acquire mesenchymal traits. This transformation is critical in cancer metastasis, as it enables cancer cells to invade surrounding tissues and migrate to distant organs. HMGB1 can interact with some receptors on cancer cells, initiating a cascade of signalling pathways that lead to the activation of some transcription factors that promote the activation of genes associated with inflammation and metastasis, including those that promote EMT [4].

HMGB1 interacts with the receptor for advanced glycation end-products RAGE and activates downstream signalling pathways such as NF- κ B, ERK, and PI3K/AKT [5]. This signalling cascade promotes the transcription of EMT-associated genes and increased expression of mesenchymal markers N-cadherin, vimentin, beta-catenin and decreased expression of epithelial markers such as E-cadherin. HMGB1-mediated EMT enhances the migration and invasion ability of cancer cells, facilitating metastasis [6]. In our study, we were focused on the effects of disruption of the HMGB1/RAGE interaction on the cell movement and invasion because this aspect is poorly understood.

Metformin is a well-established anti-diabetic medication, which has recently been used in various clinical applications, including its potential as an anti-cancer agent. Metformin specifically binds to the C-terminal acidic tail of HMGB1 and has been shown to inhibit HMGB1-induced inflammatory responses both in vitro and in vivo [7]. Based on these findings, we hypothesized that metformin's interaction with HMGB1 would interfere with its binding to the receptor for advanced glycation end products (RAGE), thereby influencing cancer metastasis.

We hypothesize that metformin inhibits HMGB1/RAGE signalling by binding to HMGB1 in competition with its receptor and reducing its extracellular availability for RAGE interaction. Targeting HMGB1 or its related pathways may serve as a therapeutic strategy to prevent EMT-driven metastasis in lung cancer.

Materials and methods. Cell culture. The human A549 lung cancer cell line was obtained from the American Type Culture Collection (ATCC, USA) and cultured in DMEM supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotic-antimycotic solution (Sigma). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂ and used between passages 2 and 8. DAPI staining confirmed the absence of Mycoplasma contamination.

MTT colourimetric assay. To assess cell viability, 3000 A549 cells were seeded per well in 96-well plates. After 24 h, cells were treated with various concentrations of metformin. Following 72 h of treatment, MTT reagent (0.5 mg/mL) was added and incubated for 3 h to allow formazan crystal formation. Crystals were solubilized with DMSO and absorbance was measured at 550 nm. Viability was normalized to untreated controls [8].

Preparation of plasmid constructs and purification of recombinant HMGB1 proteins. Recombinant HMGB1 protein was expressed in *E. coli* BL21 Poly Lys S using a pET28a+ plasmid and purified via His-tag affinity chromatography, as previously described [9].

Western blot analysis. Cells were lysed in SDS sample buffer and sonicated. Lysates were boiled, separated by 12% SDS-PAGE, and transferred to nitrocellulose membranes. After blocking in 5% BSA, membranes were incubated overnight at 4 °C with primary antibodies against RAGE (Abcam), HMGB1 (Abcam), vimentin (Cell Signalling) and β -actin (Sigma). After washing, membranes were incubated with HRP-conjugated secondary antibodies. Protein bands were visualized with chemiluminescence (Thermo Fisher) and quantified using ImageJ, with β -actin as a loading control [10].

Wound-healing assay. A549 cells were grown to confluence in 12-well plates and scratched with a pipette tip to create a wound. Cells were treated with 800 ng/mL HMGB1, 5 mM metformin, or both, in medium containing 0.1% FBS. Wound closure was imaged at 0 and 24 h using phase-contrast microscopy (Zeiss AxioVert 200M), and migration was quantified using ImageJ [11].

Immunofluorescence. Cells were seeded on glass coverslips and treated with HMGB1 (800 ng/mL) or metformin (5 mM) for 24 h. After fixation with 4% paraformaldehyde and permeabilization with Triton X-100, cells were blocked and incubated with primary antibodies against RAGE (Abcam) and vimentin (Cell Signalling) overnight at 4 °C. Alexa488-conjugated secondary antibodies and DAPI were used for visualization. Coverslips were mounted and imaged using a Zeiss AxioVert 200M epifluorescence microscope at 63 $\times$ magnification.

Statistical analysis. Data are expressed as mean $\pm$ SEM from three independent experiments. Statistical comparisons were made using one-way ANOVA (GraphPad Prism), with $p < 0.05$ considered significant.

Results. Metformin reduces A549 cell viability in a dose-dependent manner. To evaluate the cytotoxic effect of metformin on A549 cells, an MTT assay was conducted. Treatment with increasing concentrations of metformin re-

sulted in a dose-dependent reduction in cell viability. The half-maximal inhibitory concentration (IC) of metformin was established at 10.33 mM, indicating a significant inhibitory effect on A549 cell proliferation at that concentration (Fig. 1).

A549 treated with Metformin - IC50 is 10.33 mM

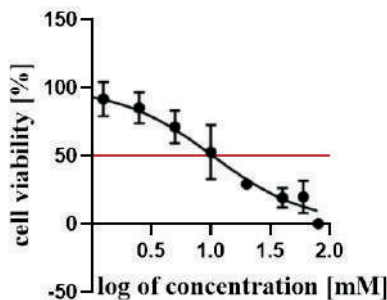


Fig. 1. MTT analysis of metformin on A549 cells. MTT assay was performed on A549 lung cancer cells treated with increasing concentrations of metformin for 72 h. Cell viability was assessed via absorbance at 550 nm and normalized to untreated control (100%). The calculated IC_{50} for metformin was 10.33 mM

Metformin inhibits HMGB1-induced cell migration in A549 cells.

To assess the effects of metformin and HMGB1 on A549 cell migration, a wound healing assay was performed under the following treatment conditions: 800 ng/mL HMGB1, 5 mM metformin, and their combination. The chosen concentration of metformin (5 mM) is non-toxic and enables a reliable evaluation of its impact on the investigated processes. Cells treated with HMGB1 alone showed increased wound closure, suggesting an enhanced migratory capacity. In contrast, metformin alone or in combination with HMGB1 significantly inhibited wound closure, indicating an anti-migratory effect. These findings suggest that metformin counteracts the pro-migratory effect of HMGB1 in A549 cells (Fig. 2).

HMGB1 enhances cytoplasmic expression of RAGE and vimentin, but metformin reduces this effect. Immunofluorescence analysis was performed to evaluate the expression patterns of RAGE and vimentin in A549 cells following treatment. HMGB1 treatment resulted in a visibly increased cytoplasmic signal for both RAGE and vimentin, indicating upregulation (Fig. 3). The signal of RAGE protein also started to localize on the cellular membrane (Fig. 3 inset) in comparison with the control treatment, indicating the activation of the RAGE signalling.

Conversely, cells treated with metformin alone or in combination with HMGB1 displayed markedly reduced cytoplasmic expression of both proteins as well as non-membrane localization of RAGE (Fig. 3). These observations were further validated by Western blot analysis. Protein levels of RAGE and vimentin were

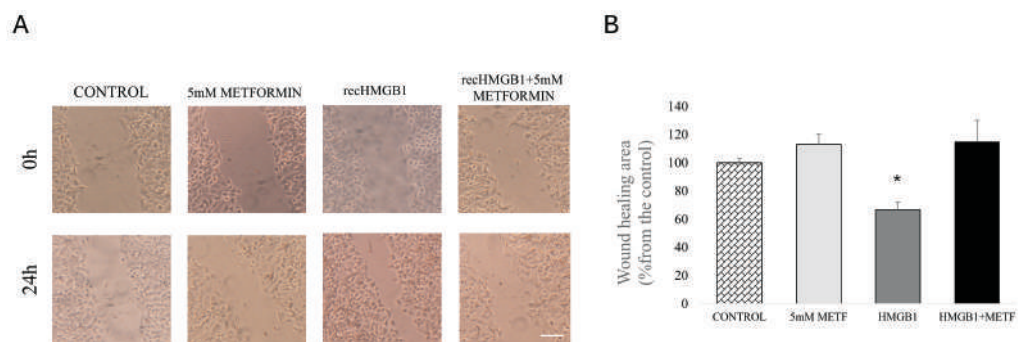


Fig. 2. Metformin inhibits HMGB1-induced A549 cell migration. (A) Representative images from wound healing assay at 0 and 24 h under the following conditions: control, 5 mM metformin, 800 ng/mL HMGB1 and HMGB1 with metformin (with 0.1% FBS in media). (B) Quantification of wound closure percentage after 24 h. Data are shown as mean $\pm$ SEM ($p < 0.05$). Scale bar: 200 μ m

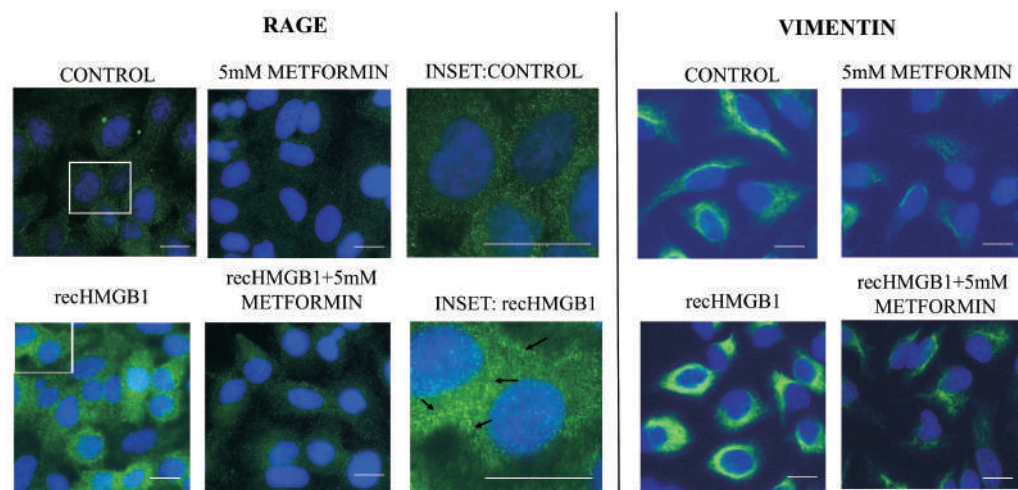


Fig. 3. HMGB1 enhances cytoplasmic expression of RAGE and vimentin, which is suppressed by metformin. Immunofluorescence staining of RAGE and vimentin in A549 cells treated with HMGB1, metformin, or both for 48 h. HMGB1 treatment increased cytoplasmic expression of both proteins, while metformin reduced expression levels, both alone and in combination with HMGB1. DAPI (blue) was used for nuclear staining. Scale bar: 20 μ m

significantly elevated in cells treated with HMGB1, while metformin alone and the combined treatment with HMGB1 resulted in reduced expression levels of both proteins, confirming the inhibitory effect of metformin on HMGB1-induced expression of mesenchymal and receptor markers (Fig. 4).

Discussion. In our previous research on triple-negative breast cancer, we demonstrated that metformin inhibits RAGE/HMGB1 pathway, thereby preventing epithelial-to-mesenchymal transition (EMT) and limiting cell migration [12].

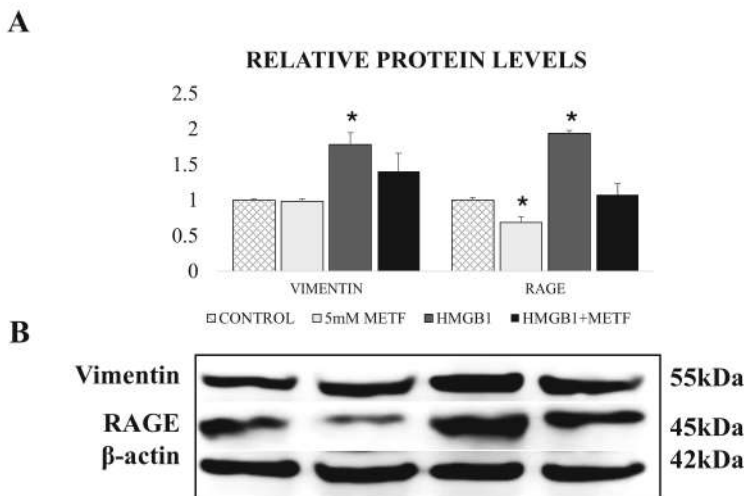


Fig. 4. Western blot analysis confirms modulation of RAGE and vimentin expression by HMGB1 and metformin. (A) Densitometric analysis of RAGE and vimentin expression normalized to β -actin. HMGB1 significantly increased expression, whereas metformin reduced its levels, even in the presence of HMGB1. Data are expressed as mean $\pm$ SEM from three independent experiments (* indicates $p < 0.05$) (B) Representative Western blot showing protein levels of RAGE, vimentin, and β -actin (loading control) in A549 cells treated with HMGB1, metformin, or both

These earlier results prompted us to explore whether a similar mechanism might be at play in lung cancer cells. In the present study, we observed that recombinant HMGB1 upregulates RAGE expression and promotes its localization to the plasma membrane. This translocation appears to be a critical step in the activation of downstream signalling pathways associated with enhanced tumour proliferation and metastatic potential. Notably, co-treatment with metformin significantly reduced both RAGE protein levels and its membrane localization. This suggests that metformin may interfere with the HMGB1/RAGE interaction, thereby disrupting subsequent pro-tumourigenic signalling.

A particularly compelling aspect of our findings is that metformin effectively suppresses HMGB1-induced EMT, as evidenced by decreased vimentin expression – a hallmark of mesenchymal transition. This reduction in mesenchymal characteristics explains why HMGB1-induced cell migration is inhibited in metformin-treated cells. Interestingly, similar EMT-inhibiting effects of metformin have been documented in other tumour types, including oral squamous cell carcinoma [13] and melanoma [14].

These data align well with a growing body of literature indicating that metformin use is associated with decreased lung cancer incidence and improved sur-

vival outcomes [15, 16]. This raises an important question: whether the suppression of the HMGB1/RAGE signalling pathway is one of the mechanisms underlying these clinical observations. Given that metformin has been reported to bind directly to HMGB1 [7], we hypothesize that it may prevent receptor activation by blocking ligand binding, thus attenuating the signalling. Taken together, our results suggest that the inhibitory effect of metformin on the HMGB1/RAGE axis may represent an important component of its broader anticancer activity in lung adenocarcinoma. These insights not only deepen our understanding of how metformin might function as an antineoplastic agent but also support its potential as an adjunctive therapeutic strategy targeting cancer progression and metastasis.

REFERENCES

- [1] NAKANO-NARUSAWA Y., M. YOKOHIRA, K. YAMAKAWA et al. (2021) Relationship between Lung Carcinogenesis and Chronic Inflammation in Rodents, *Cancers* (Basel), **13**(12), 2910, <https://doi.org/10.3390/cancers13122910>.
- [2] TANG D. R. KANG, H. J. ZEH, M. T. LOTZE (2023) The multifunctional protein HMGB1: 50 years of discovery, *Nat. Rev. Immunol.*, **23**(12), 824–841, <https://doi.org/10.1038/s41577-023-00894-6>.
- [3] CHEN Y. C., S. STATT, R. WU et al. (2016) High mobility group box 1-induced epithelial mesenchymal transition in human airway epithelial cells, *Sci. Rep.*, **6**, 18815, <https://doi.org/10.1038/srep18815>.
- [4] CHEN R., R. KANG, D. TANG (2022) The mechanism of HMGB1 secretion and release (2022) *Experimental & Molecular Medicine*, **54**(2), 91–102.
- [5] SIMS G. P., D. C. ROWE, S. T. RIETDIJK et al. (2010) HMGB1 and RAGE in inflammation and cancer, *Annu. Rev. Immunol.*, **28**, 367–388, <https://doi.org/10.1146/annurev.immunol.021908.132603>.
- [6] MITTAL V. (2018) Epithelial Mesenchymal Transition in Tumor Metastasis, *Annu. Rev. Pathol.*, **13**, 395–412, <https://doi.org/10.1146/annurev-pathol-020117-043854>.
- [7] HORIUCHI T., N. SAKATA, Y. NARUMI et al. (2017) Metformin directly binds the alarmin HMGB1 and inhibits its proinflammatory activity, *J. Biol. Chem.*, **292**(20), 8436–8446, <https://doi.org/10.1074/jbc.M116.769380>.
- [8] MOSMANN T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, *J. Immunol. Methods*, **65**, 55–63, [https://doi.org/10.1016/0022-1759\(83\)90303-4](https://doi.org/10.1016/0022-1759(83)90303-4).
- [9] ELENKOV I., P. PELOVSKY, I. UGRINOVA et al. (2011) The DNA Binding and Bending Activities of Truncated Tail-less HMGB1 protein are Differentially Affected by Lys-2 and Lys-81 Residues and Their Acetylation, *Int. J. Biol. Sci.*, **7**(6), 691–699, <https://doi.org/10.7150/ijbs.7.691>, <https://www.ijbs.com/v07p0691.htm>.
- [10] BURNETTE W. N. (1981) “Western blotting”: electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A, *Anal. Biochem.*, **112**(2), 195–203, [https://doi.org/10.1016/0003-2697\(81\)90281-5](https://doi.org/10.1016/0003-2697(81)90281-5).

- [11] RODRIGUEZ L. G., X. WU, J. L. GUAN (2005) Wound-Healing Assay. In: Guan J. L. (Ed.) Cell Migration. Methods in Molecular Biology, vol. 294, Humana Press, <https://doi.org/10.1385/1-59259-860-9:023>.
- [12] YUSEIN-MYASHKOVA S., D. VLADIMIROVA, A. GOSPODINOV et al. (2025) Metformin Inhibits Cell Motility and Proliferation of Triple-Negative Breast Cancer Cells by Blocking HMGB1/RAGE Signaling, *Cells*, **14**(8), 590, <https://doi.org/10.3390/cells14080590>.
- [13] YIN W., Y. LIU, X. LIU et al. (2021) Metformin inhibits epithelial-mesenchymal transition of oral squamous cell carcinoma via the mTOR/HIF-1 α /PKM2/STAT3 pathway, *Oncol. Lett.*, **21**(1), 31, <https://doi.org/10.3892/ol.2020.12292>.
- [14] VELOSO E. S., B. A. DE CARVALHO, F. H. DE SOUZA SILVA et al. (2022) Epithelial-mesenchymal transition inhibition by metformin reduces melanoma lung metastasis in a murine model, *Sci. Rep.*, **12**(1), 17776, <https://doi.org/10.1038/s41598-022-22235-8>.
- [15] XIAO K, F. LIU, J. LIU et al. (2020) The effect of metformin on lung cancer risk and survival in patients with type 2 diabetes mellitus: A meta-analysis, *J. Clin. Pharm. Ther.*, **45**(4), 783–792, <https://doi.org/10.1111/jcpt.13167>.
- [16] WANG Y., Y. SUN, J. HU, H. MA (2024) Clinical Effect of Treatment with Metformin for Type 2 Diabetes on Non-Small Cell Lung Cancer Patients Undergoing Immunotherapy: A Retrospective Study, *Int. J. Gen. Med.*, **17**, 6595–6604, <https://doi.org/10.2147/IJGM.S495449>.

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