Sulfisoxazole treatment does not inhibit the secretion of small extracellular vesicles

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Abstract

Recently, the study by Im et al. focused on blocking the release of EVs by cancer cells, as a strategy to block metastasis, by deploying a drug repurposing screen. Upon screening the library of FDA approved drugs in breast cancer cells in vitro, the authors reported the ability of the antibiotic Sulfisoxazole (SFX) in inhibiting EV biogenesis and secretion. The authors also reported that SFX was effective in inhibiting breast primary tumor burden and blocking metastasis in immunocompromised and immunocompetent mouse models. As we seek a compound to block EV biogenesis and secretion in our current in vivo studies, we intended to use SFX and hence performed in vitro characterization as the first step. However, treatment of three cancer cells with SFX did not reduce the amount of EVs as reported by the authors.

Over the last two decades, extracellular vesicles (EVs) have been implicated in intercellular communication and utilised as drug delivery vehicles and as reservoirs of disease biomarkers[1-5]. As they continue to garner interest, several seminal studies have established that EVs regulate various pathophysiological processes in favour of cancer progression, including remodelling the tumour microenvironment, immune evasion, coagulation, vascular leakiness, establishing the pre-metastatic niche, tropism for metastasis and transfer of chemoresistance[6-11]. Hence, there is growing interest in blocking the release of EVs and limiting their systemic circulation as a novel therapeutic avenue to treat cancer[12]. As anti-metastatic therapies are scarce, it is speculated that FDA approved drugs that target EVs could possibly fill the void.

Recently, the study by Im *et al.* focused on blocking the release of EVs by cancer cells, as a strategy to block metastasis, by deploying a drug repurposing screen[13]. The rationale was to screen the existing FDA approved library to identify drugs which can inhibit EV biogenesis or secretion with the obvious advantage of known mode of action, efficacy and toxicity profile and hence has the potential of immediate clinical utility. Upon screening the library of FDA approved drugs in metastatic breast cancer cells *in vitro*, the authors reported the ability of the antibiotic Sulfisoxazole (SFX) in inhibiting EV biogenesis and secretion. The authors also reported that SFX was effective in inhibiting breast primary tumor burden and blocking metastasis in immunocompromised and immunocompetent mouse models. SFX was proposed to target Endothelin receptor A (ETA) which is associated with EV biogenies and secretion. The findings in this study thus present SFX as a potential novel EV-targeted therapeutic alternative. As a group interested in EVs, the outcomes proposed in the study were encouraging and attractive as FDA approved drugs targeting EV release are limited. Recently, Datta *et al.* also performed a repurposing screen to identify drugs that modulate the release of EVs in prostate cancer cells[12]. However, the identified drugs are yet to be tested *in vivo*.

As we seek a compound to block EV biogenesis and secretion in our current *in vivo* studies, we intended to use SFX and hence performed *in vitro* characterization as the first step. However, treatment of 4T1 breast cancer cells with SFX did not reduce the amount of EVs as reported by the authors. We acknowledge the fact that our EV isolation protocol was different from the study and hence could have attributed to the

varied results. In order to rule out the possibility of variations in the method of EV isolation or cell-type dependency, we exactly followed the protocol employed by the authors to isolate EVs from three different cell types (4T1, MDA-MB231 and C26), two of which were used by the authors. Consistent with our previous observations, treatment of the cancer cells with varying concentrations of SFX (50, 100 and 200 µM) did not impede the release of EVs while the positive control ceramide inhibitor GW4869, at very low concentration (5 µM), inhibited EV secretion. Upon EV isolation, we quantified the protein amount, particle number and performed western blotting for EV markers (TSG101, Alix), all normalised to equal cell number (**Fig. 1**). Contrary to the authors claim of SFX treatment led to a 3-fold decline in EV particle number, we observed a significant increase in particle number upon SFX (200 µM) treatment.

Overall, we report that SFX does not reduce the release of EVs and emphasise caution in using SFX as a drug to block EV release. However, we do acknowledge the fact that our findings do not challenge the authors main conclusion of SFX mediated reduction of primary tumour burden and metastasis though our results suggest that the phenotype observed may not be cancer cell-derived EV mediated. Further research is needed to understand as how SFX can reduce primary tumor burden and inhibit metastasis.

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Competing interests

The authors declare no competing financial interests.

References

1 Peinado, H. *et al.* Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat. Med.* **18**, 883-891 (2012).

2 Kalra, H. *et al.* Extracellular vesicles containing oncogenic mutant beta-catenin activate Wnt signalling pathway in the recipient cells. *J Extracell Vesicles* **8**, 1690217, doi:10.1080/20013078.2019.1690217 (2019).

3 Kamerkar, S. *et al.* Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature* **546**, 498-+, doi:10.1038/nature22341 (2017).

4 Yoshioka, Y. *et al.* Ultra-sensitive liquid biopsy of circulating extracellular vesicles using ExoScreen. *Nature communications* **5**, 3591, doi:10.1038/ncomms4591 (2014).

5 Thery, C. *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* **7**, 1535750, doi:10.1080/20013078.2018.1535750 (2018).

6 Tkach, M. & Thery, C. Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. *Cell* **164**, 1226-1232, doi:10.1016/j.cell.2016.01.043 (2016).

7 Al-Nedawi, K. *et al.* Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat. Cell Biol.* **10**, 619-624 (2008).

8 Rajesh, D., Schell, K. & Verma, A. K. Ras mutation, irrespective of cell type and p53 status, determines a cell's destiny to undergo apoptosis by okadaic acid, an inhibitor of protein phosphatase 1 and 2A. *Mol. Pharmacol.* **56**, 515-525 (1999).

9 Hoshino, A. *et al.* Tumour exosome integrins determine organotropic metastasis. *Nature* **527**, 329-335 (2015).

10 Kalluri, R. & LeBleu, V. S. The biology, function, and biomedical applications of exosomes. *Science* **367**, doi:10.1126/science.aau6977 (2020).

11 Anand, S., Samuel, M., Kumar, S. & Mathivanan, S. Ticket to a bubble ride: Cargo sorting into exosomes and extracellular vesicles. *Biochim Biophys Acta Proteins Proteom*, doi:10.1016/j.bbapap.2019.02.005 (2019).

12 Datta, A. *et al.* High-throughput screening identified selective inhibitors of exosome biogenesis and secretion: A drug repurposing strategy for advanced cancer. *Sci Rep* **8**, 8161, doi:10.1038/s41598-018-26411-7 (2018).

13 Im, E. J. *et al.* Sulfisoxazole inhibits the secretion of small extracellular vesicles by targeting the endothelin receptor A. *Nature communications* **10**, 1387, doi:10.1038/s41467-019-09387-4 (2019).

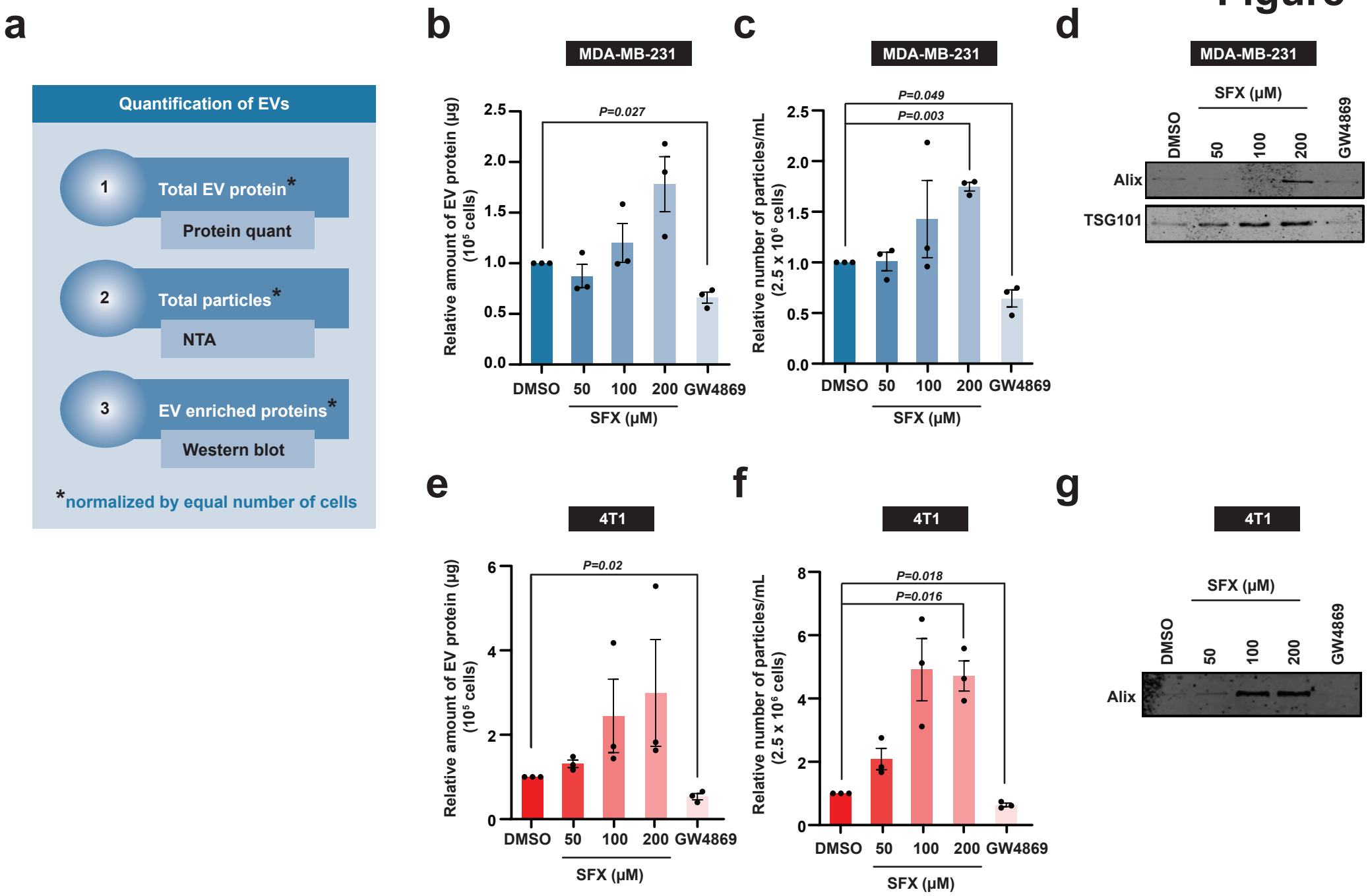
Figure legend

Figure 1

Quantification of EVs released by cells with or without SFX and GW4869

(a) Schematic of EV quantification by three different methods. Firstly, the total EV protein amount was quantified and normalised to equal number of live cells. Secondly, nanoparticle tracking analysis (NTA) was performed to quantify the total number of particles normalised to equal number of live cells. Lastly, Western blot analysis of EV samples obtained from equal number of live cells was performed for EV enriched proteins. **(b)** Relative amount of EV protein normalised to 10^6 MDA-MB-231 cells is shown. **(c)** Relative number of particles normalised to 10^6 MDA-MB-231 cells is depicted. **(d)** Western blot analysis of EV enriched proteins Alix and TSG101 in EV samples obtained from 10^6 MDA-MB-231 cells. **(e)** Relative amount of EV protein normalised to 10^6 4T1 cells is shown. **(f)** Relative number of particles normalised to 10^6 4T1 cells is depicted. **(g)** Western blot analysis of EV enriched proteins Alix in EV samples obtained from 10^6 4T1 cells. All data are represented as mean ± s.e.m. statistical significance was determined by paired two-tailed t-test.

Figure 1


a
Quantification of EVs
1
Total EV protein*
Protein quant
2
Total particles*
NTA
3
EV enriched proteins*
Western blot
*normalized by equal number of cells
b
MDA-MB-231
Relative amount of EV protein (µg) (10⁵ cells)
P=0.027
DMSO 50 100 200 GW4869
SFX (µM)
c
MDA-MB-231
Relative number of particles/mL (2.5 x 10⁶ cells)
P=0.049
P=0.003
DMSO 50 100 200 GW4869
SFX (µM)
d
MDA-MB-231
SFX (µM)
DMSO 50 100 200 GW4869
Alix
TSG101
e
4T1
Relative amount of EV protein (µg) (10⁵ cells)
P=0.02
DMSO 50 100 200 GW4869
SFX (µM)
f
4T1
Relative number of particles/mL (2.5 x 10⁶ cells)
P=0.018
P=0.016
DMSO 50 100 200 GW4869
SFX (µM)
g
4T1
SFX (µM)
DMSO 50 100 200 GW4869
Alix