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Original Articles

Natural compound Tetrocarcin-A downregulates Junctional Adhesion Molecule-A in conjunction with HER2 and inhibitor of apoptosis proteins and inhibits tumor cell growth

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ABSTRACT

Overexpression of the tight junction protein Junctional Adhesion Molecule-A (JAM-A) has been linked to aggressive disease in breast and other cancers, but JAM-targeting drugs remain elusive. Screening of a natural compound library identified the antibiotic Tetrocarcin-A as a novel downregulator of JAM-A and human epidermal growth factor receptor-2 (HER2) protein expression in breast cancer cells. Lysosomal inhibition partially rescued the downregulation of JAM-A and HER2 caused by Tetrocarcin-A, and attenuated its cytotoxic activity. Tetrocarcin-A treatment or JAM-A silencing reduced AKT and ERK phosphorylation, inhibited c-FOS phosphorylation at Threonine-232 (its transcriptional regulation site), inhibited nuclear localization of c-FOS, and downregulated expression of the inhibitor of apoptosis proteins (IAP). This was accompanied by Tetrocarcin-A-induced caspase-dependent apoptosis. To begin evaluating the potential clinical relevance of our findings, we extended our studies to other models. Encouragingly, Tetrocarcin-A downregulated JAM-A expression and caused cytotoxicity in primary breast cells and lung cancer stem cells, and inhibited the growth of xenografts in a semi-*in vivo* model involving invasion across the chicken egg chorioallantoic membrane. Taken together, our data suggest that Tetrocarcin-A warrants future evaluation as a novel cancer therapeutic by virtue of its ability to downregulate JAM-A expression, reduce tumorigenic signaling and induce apoptosis.

1. Introduction

A major challenge in breast cancer treatment is to identify biomarkers and therapeutic targets for metastasis, the main cause of patient death. One target which has proven valuable is the human epidermal growth factor receptor-2 (HER2), which is overexpressed in 20–25% of invasive breast cancers and marks a particularly aggressive form of the disease [1,2]. We recently showed that the tight junction protein Junctional Adhesion Molecule-A (JAM-A) acts as a novel regulator of HER2 protein degradation and signaling in breast cancer cells [3]. JAM-A is expressed on epithelial and endothelial cells, and plays important physiological roles. In the pathophysiological setting of cancer, however, high expression of JAM-A has been linked to aggressive disease and poor outcome in breast cancer [4], head and neck carcinomas [5], non-small cell lung cancer [6], cervical adenocarcinoma [7], nasopharyngeal carcinoma [8] and multiple myeloma [9]. Intriguing recent evidence also suggests that JAM-A is required for the maintenance of cancer stem cells in various settings [10,11].

Given the links between JAM-A expression and tumor aggressiveness, plus pre-clinical data that JAM-A antagonism/downregulation stalls murine tumor growth [12] and induces apoptosis in breast cancer cells [13], the present study sought to identify anti-cancer compounds that downregulate JAM-A expression. Screening of a National Cancer Institute natural compound library [14] on JAM-A-overexpressing versus control breast cancer cells revealed promising bioactivity of a compound termed Tetrocarcin-A. Tetrocarcin-A is an antibiotic isolated from *Micromonospora chalcea* subsp. *Kazunoensis* [15], which is active against Gram-positive bacteria but has also been shown to inhibit AKT [16] and Bcl2 and to induce apoptosis in cancer cells [17]. Lack of

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expression of Bcl-2 or AKT family members in bacteria suggests that the antibacterial properties of Tetrocarcin-A are unrelated to their activity against Bcl-2 [18] or AKT. Supporting the potential importance of these signaling events, Tetrocarcin-A has been shown to inhibit growth of several tumor types in *in vivo* mouse models [19]. However the upstream signaling events leading to Bcl2 or AKT inhibition and apoptosis are unknown. Here we show for the first time that Tetrocarcin-A induces protein-level downregulation of JAM-A in conjunction with HER2 and its downstream effectors phospho-ERK1/2 and -AKT in breast cancer cells, at least in part by a lysosomal degradation pathway. This is accompanied by reduced phosphorylation of the transcription factor c-FOS on Threonine-232 (the residue controlling its transcriptional activity), inhibition of c-FOS nuclear localization, downregulation of inhibitor of apoptosis proteins (IAP), and the induction of apoptosis. Encouragingly, the anti-growth effects of Tetrocarcin-A occurred in primary breast cancer cells, lung cancer stem cells and a semi-*in vivo* model of xenograft invasion across the chicken egg chorioallantoic membrane. Taken together, our data suggest value in investigating Tetrocarcin-A as a novel cancer therapeutic by virtue of its ability to downregulate JAM-A expression, induce apoptosis and reduce tumorigenic signaling.

2. Materials and methods

2.1. Cells

MCF7-HER2, MCF 10A [3], BT474 Trastuzumab-resistant (BT474-Tr) [20,21] and SK-BR-3 [22] breast cancer cells were cultured as described. MCF7 cells stably expressing full length JAM-A or pcDNA3 empty vector (plasmids a kind gift of C.A. Parkos, University of Michigan, cell line made by K. Brennan); were cultured in MEM media plus usual additives. Primary breast cell cultures were generated and cultured as described [23] from symptomatic patients undergoing breast cancer surgery in Beaumont Hospital, with informed consent and ethical approval (Beaumont Hospital Medical Ethics (Research) Committee). Lung cancer stem cells isolated from surgical specimens were cultured as previously described [24].

2.2. Transfections

Gene expression of JAM-A, HER2 or c-FOS was transiently silenced in MCF7-HER2 cells by transfection with 25 nM or 50 nM siRNA as indicated in figure legends (siJAM1 CCTTCTAAGTAGACAGCAA, siJAM11 CGGGGGUCGCAGGAAUCUG; HER2 siGenome SMARTpool, M-003126-4; c-FOS SMARTpool: ON-TARGETplus L-003265-00-0005 Dharmacon). Non-targeting siRNA (siNEG) was used as a control (siGENOME non-targeting siRNA#1, Dharmacon). Transfections were carried out using Dharmafect-1 or Lipofectamine-2000 transfection reagent (Dharmacon/Thermo Scientific).

2.3. Cell growth assays

5000 MCF7-HER2 or breast primary cells were plated in triplicate wells of 96-well plates, while cancer stem cells were plated at 5000/well in triplicate wells of non-adherent 96-well plates. Cells were treated with 50 nM siJAM-A or Tetrocarcin-A at the doubling time of MCF7-HER2 cells (38h). At the indicated time points, Alamar Blue (Sigma) was diluted in culture medium (1:10), whereupon 100 µL was added to wells, incubated at 37 °C/3h and spectrophotometrically quantitated at 570 nm on a VICTOR™ X3 Multilabel Plate Reader (Perkin Elmer). For cancer stem cells, Alamar Blue was incubated for 24 h at 37 °C.

2.4. Colony forming assays

MCF7-HER2 cells were plated in 6-well plates and treated at their doubling time with 2.5 µM Tetrocarcin-A or vehicle (0.01% v/v DMSO). Culture medium was exchanged a day later with drug-free medium, and cells stained on day 10 with 0.6% (w/v) crystal violet (Sigma).

2.5. Electrophoresis and western blot analysis

Following silencing of JAM-A or HER2/treatment with 2.5 µM Tetrocarcin-A or the lysosomal inhibitor chloroquine dissolved in H_2O (25 µM), cell extracts were prepared and western blotting was performed as described [3] for human JAM-A, Caspase 9, Bcl2 (BD Biosciences), HER2, p53, pERK1/2, Total ERK1/2, p-AKT-S473, p-AKT-T308, Total AKT, cIAP-1, XIAP-2, (Cell Signaling), p-c-FOS-T232 or β-actin (Abcam), Bcl-xL (Biolegend), cIAP-2 and survivin (R&D Systems). To determine the nuclear versus non-nuclear localization of c-FOS following manipulation of JAM-A levels, MCF7-HER2 cells were first treated for 40h with 2.5 µM Tetrocarcin-A or vehicle (0.01% v/v DMSO); or gene-silenced for JAM-A (72h). Subsequently, nuclear versus non-nuclear fractions were isolated using EpiQuik Nuclear Extraction Kits as per manufacturer's instructions (EpiGenetek) and Western blotted for c-FOS (Santa Cruz), the cytoplasmic marker GAPDH (Santa Cruz) or the nuclear marker Lamin A/C (Cell Signaling). In all blots, HRP-conjugated secondary anti-rabbit (Cell Signaling) or anti-mouse (Sigma) IgG was used to detect primary antibodies, and blots were developed using Western Lightning enhanced chemiluminescence solution (Perkin Elmer) on a Chemidoc system (Bio-Rad). Image Lab software (Bio-Rad) was used for densitometric quantification of western blots, using β-actin as a loading control for whole cell lysate experiments. The average results of 3 independent blots were graphed along with the standard error of the mean values.

2.6. Apoptosis assays

MCF7-HER2 cells were plated in 6-well plates and treated at their doubling time with 2.5 µM Tetrocarcin-A or vehicle (0.01% v/v DMSO). Cells were stained for Annexin V/propidium iodide (PI) as described [25]. In parallel, MCF7-HER2 cells were plated in 24-well plates and treated at their doubling time with 2.5 µM Tetrocarcin-A or vehicle (0.01% v/v DMSO). Cells were treated 1h later with the pan-caspase inhibitor zVAD-FMK (50 µM; R&D Systems) or vehicle control (0.01% v/v DMSO). Cells were stained with PI and analysed flow cytometrically on a BD FACS-Calibur flow cytometer (BD Biosciences). For analysing loss of mitochondrial membrane potential, cells were treated with 30 nM tetramethylrhodamine methyl ester perchlorate (TMRM) (Sigma) and flow cytometrically analysed.

2.7. Chorioallantoic membrane assay and immunohistochemistry (IHC)

Semi-*in vivo* chorioallantoic membrane assays were performed as described [26,27]. Briefly, 2×10^6 MCF7-HER2 breast cancer cells were resuspended in 25 µL serum-free medium/25 µL Matrigel (BD Biosciences) and implanted on the chorioallantoic membrane of fertilized chicken eggs on day 8 of gestation. Xenografts were topically treated with 2.5 µM Tetrocarcin-A or vehicle (0.01% DMSO in 15 µL PBS) from days 10–13 inclusive. Xenografts and their surrounding chorioallantoic membrane were sampled on day 13, fixed in 4% (w/v) paraformaldehyde, paraffin-embedded and cut into 5 µm sections. Sections were deparaffinised and stained for anti-human JAM-A (Abnova), Ki67 (Mib1) and cytokeratin (CAM-5; BD Biosciences). Representative gross and microscopic images (scale bar, 25 µm) are shown, in addition to the percentage of cells negative for Ki67 based on 500 cells in intact tumor islands adjacent to the CAM.

2.8. Statistical analysis

Two-tailed unpaired student's *t*-tests were used for statistical testing, as detailed in individual figure legends.

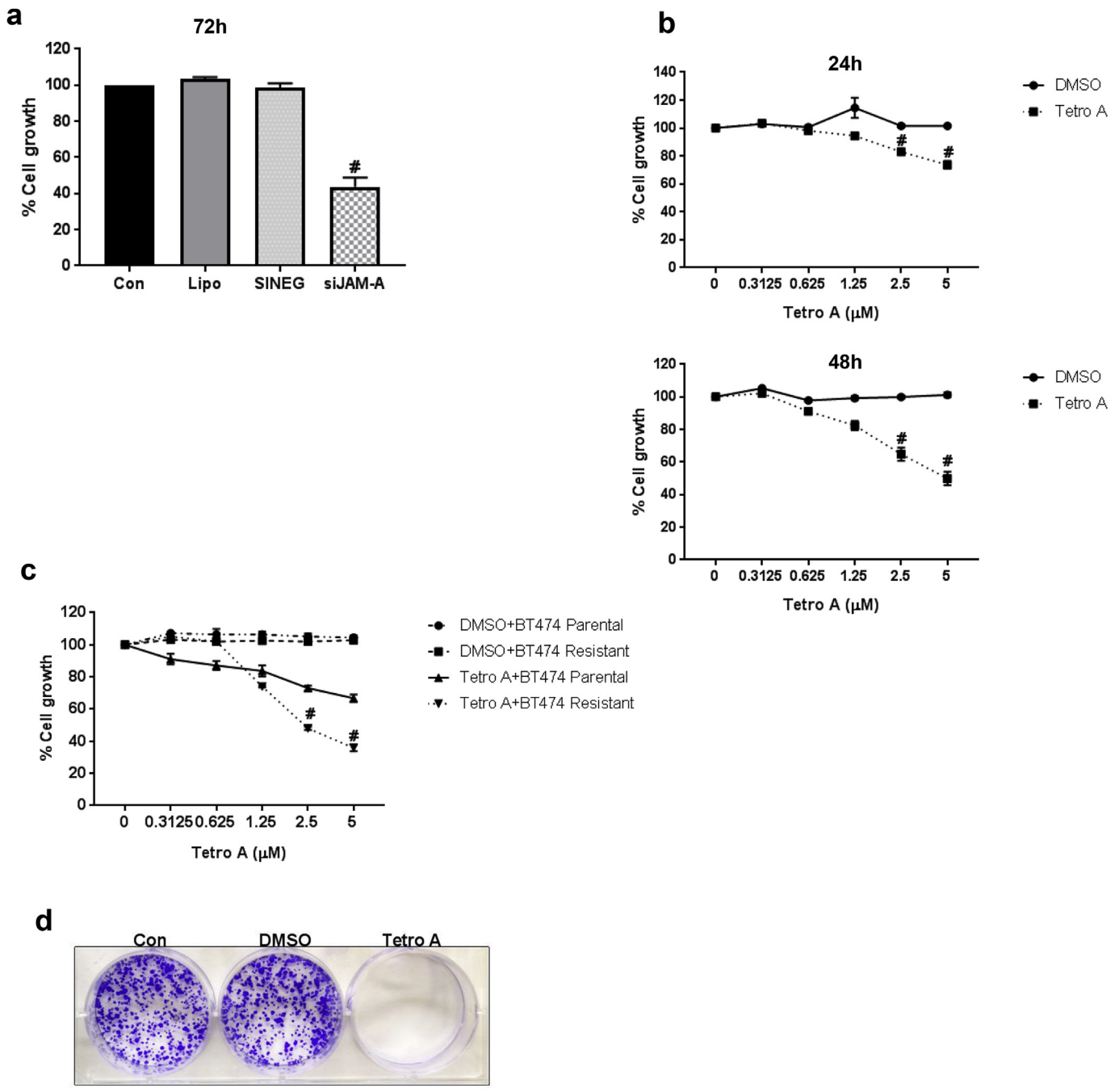


Fig. 1. Tetrocarcin-A reduces cell growth and inhibits colony-forming ability of breast cancer cells. MCF7-HER2 cells (a,b) or BT474 parental and Trastuzumab-resistant cells (c) were plated in 96-well plates and treated with 50 nM siJAMA-A or non-targeting siRNA (siNEG) after 24h or at their doubling time with the indicated concentrations of Tetrocarcin-A (The NCI/DTP Open Chemical Repository) or vehicle (0.01% v/v DMSO). Cell growth was assessed at 24 and 48h (b,c) or 72h (a) by Alamar Blue assay. (d) MCF-HER2 cells were plated in 6-well plates and treated at their doubling time with 2.5 μM Tetrocarcin-A or vehicle (0.01% v/v DMSO) for 24h and stained on day 10 with 0.6% (w/v) crystal violet. Error bars refer to standard error of the mean of triplicate experiments (#$p < 0.05$ by two-tailed unpaired student's *t*-test; comparing vehicle control vs Tetrocarcin-A (Fig. 1b) or Tetrocarcin-A-treated parental versus Trastuzumab-resistant BT474 cells (Fig. 1c). All experiments were independently repeated three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Tetrocarcin-A inhibits the growth of breast cancer cells

We and others have reported correlations between high JAM-A expression and aggressive disease in breast cancer patients [3,4,13]. This was confirmed in a gene expression dataset of 137 patients in the Oncomine database [28], in which JAM-A mRNA expression was 2.5-fold higher than that in normal breast tissue (Supplementary Fig.1). Since gene silencing of JAM-A reduced the growth of MCF7 breast cancer cells overexpressing HER2 (MCF7-HER2) [29] (Fig. 1a); we screened a natural compound library for altered bioactivity in JAM-A-overexpressing versus control cells (data not shown). The antibiotic Tetrocarcin-A exerted concentration- and time-dependent reductions in the growth of MCF7-HER2 cells (Fig. 1b) and concentration-dependent growth reductions in Trastuzumab-sensitive and -resistant BT474 cells

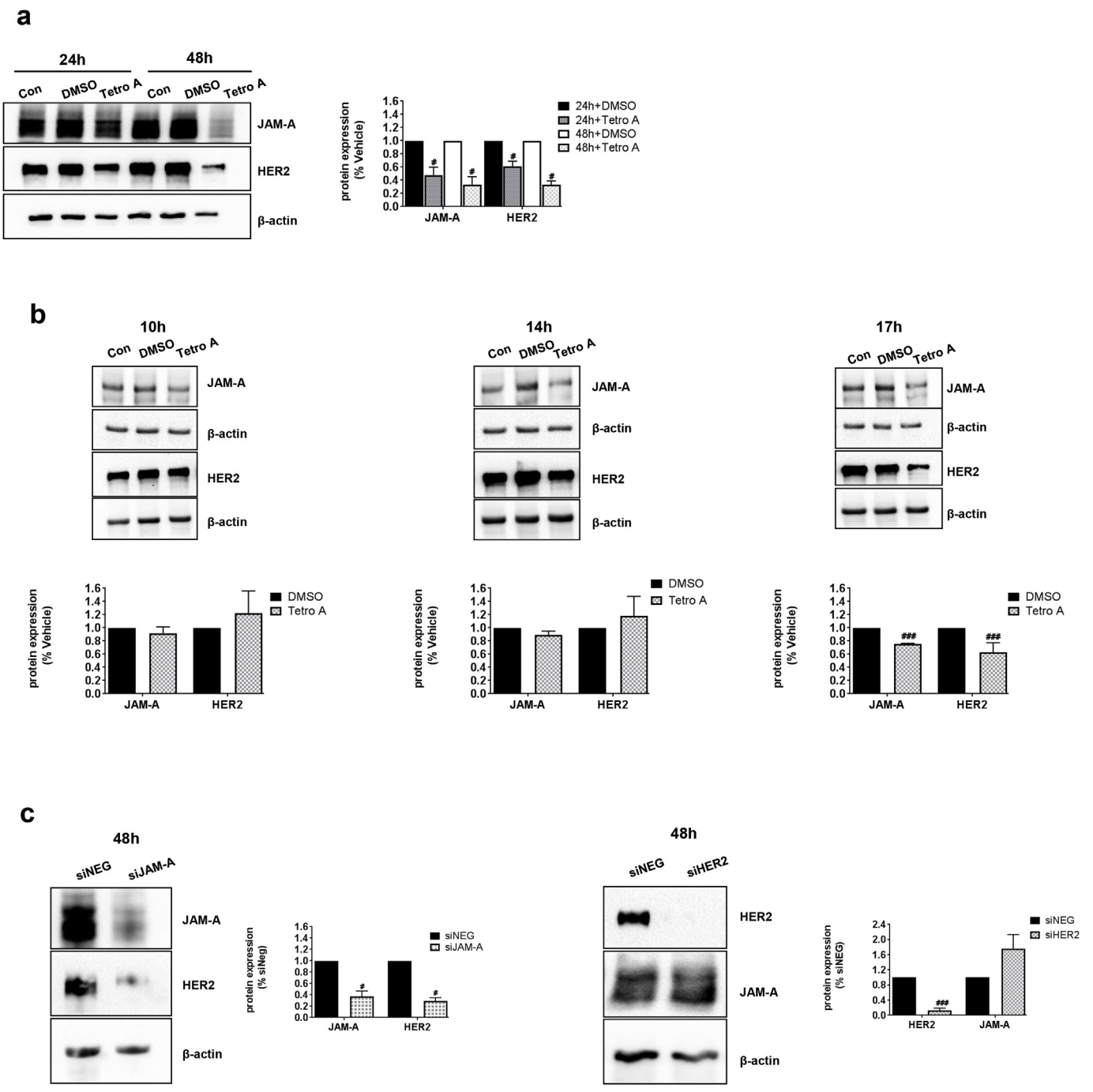


Fig. 2. Tetrocarcin-A downregulates JAM-A and HER2 protein expression in breast cancer cells. MCF7-HER2 cells were seeded in 6-well plates and treated at their doubling time with 2.5 μM Tetrocarcin-A (The NCI/DTP Open Chemical Repository) or vehicle (0.01% v/v DMSO). Cell extracts were prepared at 24 or 48h (a) or 10, 14, 17h (b) and Western blotted for human JAM-A, HER2 and β-actin. (c) MCF7-HER2 cells were transfected with 25 nM JAM-A siRNA, HER2 siRNA or non-targeting siRNA (siNEG). Cell extracts were prepared at 48h and western blotting performed for human JAM-A, HER2 and β-actin. Error bars refer to standard error of the mean of triplicate experiments (#p < 0.05; ###p < 0.005, by two-tailed unpaired student's *t*-test; comparing vehicle control vs Tetrocarcin-A; siNEG vs siJAM-A or siHER2). All experiments were independently repeated three times.

[20,21] (Fig. 1c: parental, resistant respectively). Tetrocarcin-A also inhibited long-term survival of MCF7-HER2 cells in colony-forming assays (Fig. 1d) despite its withdrawal after only 24 h treatment in a 10-day period. A further illustration of Tetrocarcin-A bioactivity under stringent conditions was the fact that drug treatment was initiated after a population doubling, when cells are less sensitive to chemotherapeutic drugs [30]. In addition, a sublethal concentration of Tetrocarcin-A (1 μM) sensitized MCF7-HER2 cells to the anti-growth effects of cisplatin (Supplementary Fig.2a). Taken together, these data suggest that Tetrocarcin-A has anti-proliferative properties. It must be noted however that the drug also exerted a concentration-dependent anti-growth effect on the non-transformed normal-like cell line MCF 10A (Supplementary Fig.2b), in common with many cancer drugs which also affect normal cells.

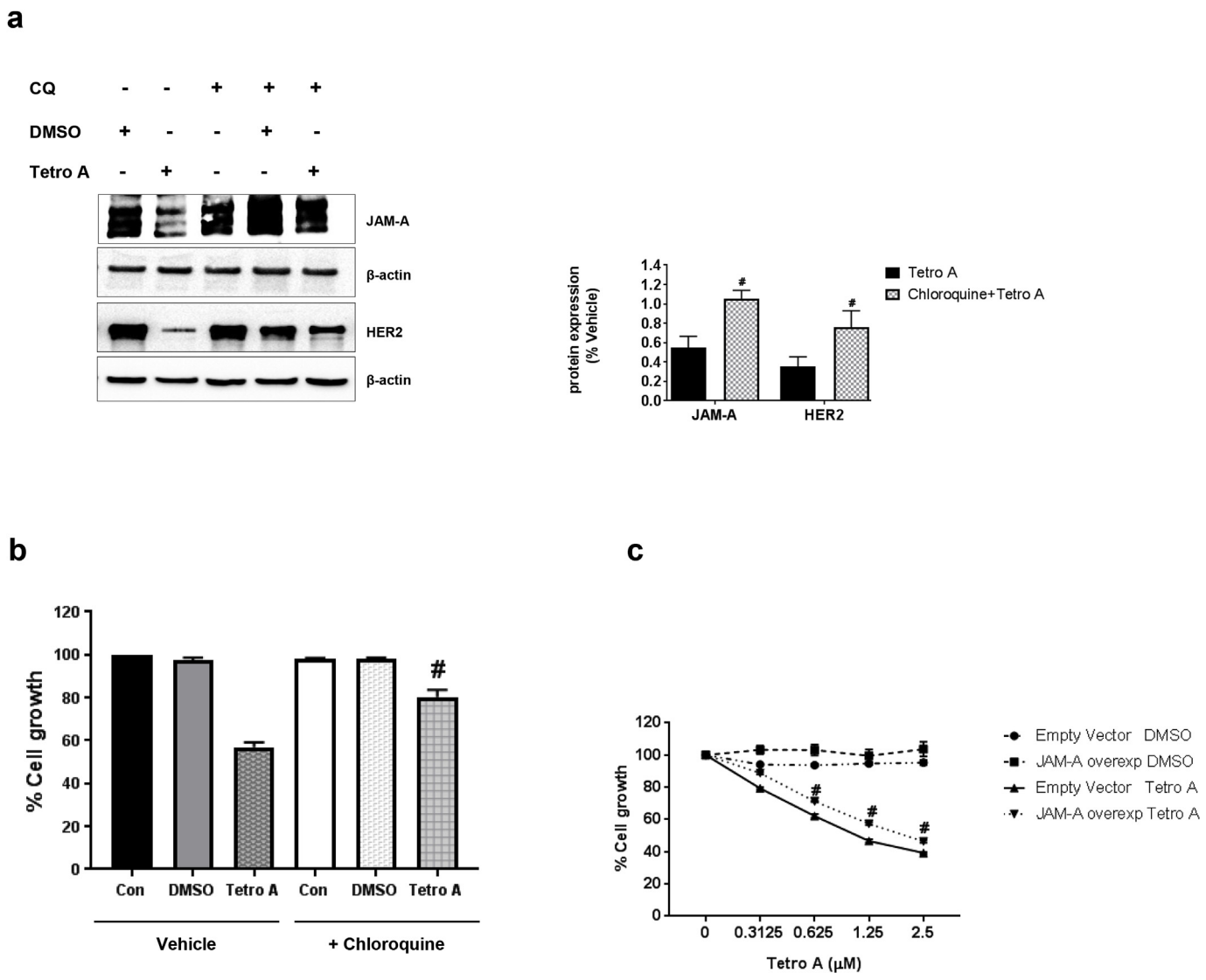


Fig. 3. Chloroquine partially rescues JAM-A and HER2 expression and attenuates cell growth defects in Tetrocarcin-A-treated breast cancer cells. (a,b) MCF7-HER2 cells were pretreated after their doubling time with 2.5 μM Tetrocarcin-A (The NCI/DTP Open Chemical Repository) or vehicle (0.01% v/v DMSO). After 3h, cells were treated with 25 μM of the lysosomal inhibitor chloroquine (CQ), whereupon cellular extracts were prepared or Alamar Blue assays performed 42h after Tetrocarcin-A treatment. Western blotting was performed for human JAM-A, HER2 or β-actin. Error bars refer to standard error of the mean of triplicate experiments (#$p < 0.05$; by two-tailed unpaired student's *t*-test, comparison made between Tetrocarcin-A treated cells vs Tetrocarcin-A + chloroquine-treated cells. All experiments were repeated three times and representative blots shown. (c) MCF7-JAM-A overexpressing cells or pcDNA3 empty vector controls were seeded in 96-well plates-and treated with Tetrocarcin-A or vehicle (0.01% v/v DMSO) and cell growth assessed at 48h by Alamar Blue assay. Error bars refer to standard error of the mean of triplicate experiments (#$p < 0.05$; by two-tailed unpaired student's *t*-test, comparison made between empty vector-overexpressing cells treated with Tetrocarcin-A vs JAM-A- overexpressing cells treated with equivalent concentrations of Tetrocarcin-A. All experiments were independently repeated three times.

3.2. Tetrocarcin-A downregulates JAM-A and HER2 protein expression in breast cancer cells via a pathway involving lysosomes

We next investigated the effects of Tetrocarcin-A on the expression of JAM-A and potential downstream molecules (Figs. 2 and 3). As shown in Fig. 2a, Tetrocarcin-A exerted time-dependent reductions in the protein expression of both JAM-A and HER2 in MCF7-HER2 cells. Since downregulation of both molecules commenced around 17h after treatment (Fig. 2b), we questioned if levels of one could regulate that of the other. Transient gene silencing studies confirmed our previous findings [3] that HER2 expression levels are sensitive to alterations in JAM-A expression, while JAM-A expression is not affected by HER2 silencing (Fig. 2c).

In investigating the mechanisms whereby Tetrocarcin-A might reduce JAM-A expression, we found that the lysosomal inhibitor chloroquine partially rescued the downregulation of JAM-A and HER2 caused by Tetrocarcin-A (Fig. 3a) and concurrently attenuated its cytotoxicity (Fig. 3b). Taken together, our results suggest that downregulation of JAM-A and HER2 levels, at least in part through a lysosomal pathway, contribute to the cytotoxicity of Tetrocarcin-A. This was supported by a small (but statistically significant) protective effect against Tetrocarcin-induced reductions in cell growth of MCF7 cells overexpressing JAM-A versus empty vector (Fig. 3c).

3.3. Tetrocarcin-A represses the potential transcriptional activity of c-FOS

Since JAM-A has been demonstrated to activate MAPK [31,32] and PI3K [8] signaling pathways, we next investigated potential alterations in these pathways downstream of Tetrocarcin-A treatment. Tetrocarcin-A induced time-dependent reductions in both ERK1/2 (Fig. 4a) and AKT (Serine-473 & Threonine-308), Fig. 4b) phosphorylation in MCF7-HER2 cells which mirrored those caused by JAM-A silencing. However, since

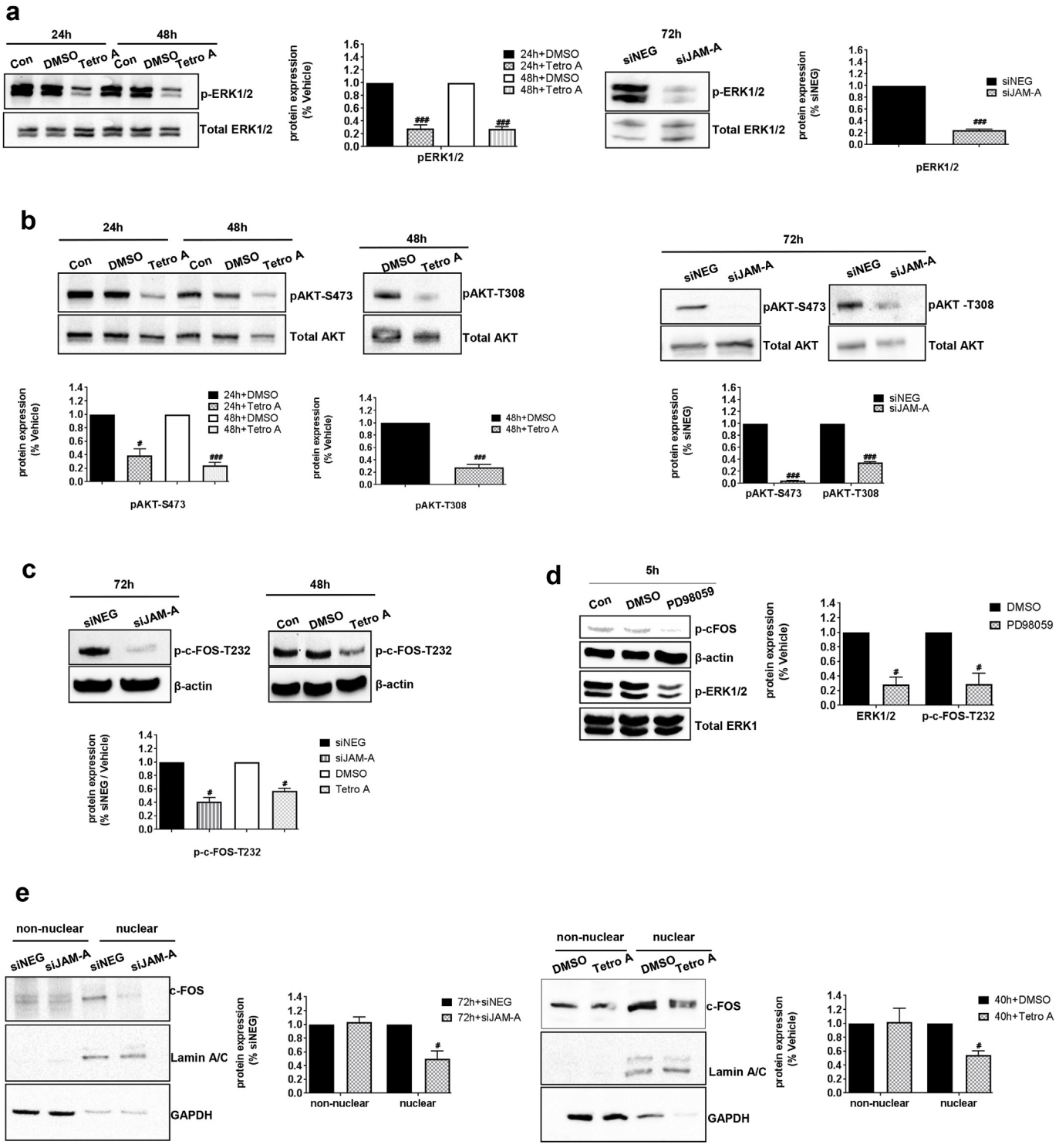


Fig. 4. Tetrocarcin-A represses the potential transcriptional activity of c-FOS through a pathway likely involving MAPK signaling. (a,b) MCF7-HER2 cells were seeded in 6-well plates and treated at their doubling time with 2.5 μM Tetrocarcin-A or vehicle (0.01% v/v DMSO). Cell extracts were prepared at 24 and 48h. Western blotting was performed for human pERK1/2, total ERK1/2, p-AKT (Ser-478 and Thr-308) and total AKT. In parallel, cells were transfected for 72h with siRNA to JAM-A or non-targeting siRNA (siNEG) and the same proteins blotted for. (c,d) MCF7-HER2 cells were seeded in 6-well plates and treated at their doubling time with 2.5 μM Tetrocarcin-A, 50 μM PD98059 or vehicle (0.01%v/v DMSO for Tetrocarcin-A and 3.5% v/v DMSO for PD98059). Cell extracts were prepared at 5 and 48h. Western blotting was performed for human pERK1/2, total ERK1/2, p-c-FOS-T232 or β-actin. In parallel, cells were transfected for 72h with siRNA to JAM-A or non-targeting siRNA (siNEG) and p-c-FOS-T232 blots performed. Experiments were independently repeated three times, and representative blots shown. (e) MCF7-HER2 cells were seeded in 100 mm plates and treated at their doubling time with 2.5 μM Tetrocarcin-A or vehicle (0.01% v/v DMSO for Tetrocarcin-A). In parallel MCF7-HER2 cells plated in 100 mm plates were transfected for 72h with siRNA to JAM-A or non-targeting siRNA (siNEG). Nuclear and non-nuclear cellular fractions were isolated at 40h (Tetrocarcin-A-treated) or 72h (JAM-silenced). Western blotting was performed for human c-FOS, Lamin A/C or GAPDH. Error bars refer to standard error of the mean of triplicate experiments (#$p < 0.05$; ###$p < 0.005$, by two-tailed unpaired student's *t*-test; comparing vehicle control vs Tetrocarcin-A or siNEG vs siJAM-A). Experiments were independently repeated three times and representative blots shown.

HER2 also regulates MAPK [33] and PI3K [34] signaling, Tetrocarcin-induced reductions in AKT/ERK phosphorylation may also reflect HER2 reductions secondary to JAM-A loss.

MAPK pathway signaling activates c-FOS transcriptional activity [35,36], which in tumor cells drives a program of gene expression controlling cell motility, invasiveness, proliferation and apoptosis [37,38]. As shown in Fig. 4c, JAM-A loss (induced either by transient gene silencing or Tetrocarcin-A treatment) reduced Threonine-232-phosphorylated c-FOS levels similarly to that observed with the MEK/ERK inhibitor PD98059 (Fig. 4d) and inhibited c-FOS nuclear localization in MCF7-HER2 cells (Fig. 4e). Since c-FOS is transcriptionally activated upon phosphorylation at Threonine-232 [39], and Tetrocarcin-A reduced T-232 phosphorylation and inhibited c-FOS nuclear localization, it is likely that Tetrocarcin-A-induced reductions in T-232-c-FOS phosphorylation would impact breast cancer cell proliferation [40] by repressing c-FOS transcriptional activity.

3.4. Tetrocarcin-A induces caspase-dependent apoptosis in breast cancer cells

Since reduced AKT and MAPK signaling have been linked to apoptosis [41], we sought to test if cell growth reductions in response to Tetrocarcin-A reflected cellular apoptosis. Tetrocarcin-A induced time-dependent upregulations in p53 protein expression (Fig. 5a), which is consistent with the pro-apoptotic (tumor suppressive) functions of p53 being activated upon exposure to environmental or oncogenic stress [42]. Accordingly, dual Annexin V/propidium iodide (PI) staining revealed increased apoptosis in Tetrocarcin-A-treated samples compared to controls (Fig. 5b). This was accompanied by activation of caspase-9 and -7, as evidenced by reduced levels of both procaspases in conjunction with conversion into the caspase-7 active fragment p20 (Fig. 5c). The involvement of caspases in Tetrocarcin-A-induced apoptosis was then confirmed using the broad-range caspase inhibitor zVAD.FMK. Specifically, co-incubation of Tetrocarcin-A-treated cells with zVAD.fmk significantly attenuated the number of PI-positive cells (Fig. 5d) relative to Tetrocarcin-A treatment alone. Since pro-caspase-9 levels decreased downstream of Tetrocarcin-A treatment, we also investigated loss of mitochondrial membrane potential (which acts downstream of the mitochondrial pathway of apoptosis) [43]. Tetrocarcin-A induced a significant loss of mitochondrial membrane potential (Fig. 5e), confirming its role as a pro-apoptotic agent. Furthermore Tetrocarcin-A reduced the protein expression of the apoptosis inhibitor XIAP (Fig. 5f), a potent inhibitor of apoptosis shown to be aberrantly expressed in different cancers and to confer resistance to some anti-cancer therapeutics [44]. Tetrocarcin-A also reduced the protein levels of cIAP-1, cIAP-2 and survivin (Fig. 5f), but had no effect on the expression of Bcl2 or Bcl-xL (Fig. 5g).

Having shown that Tetrocarcin-A inhibits c-FOS nuclear localization and reduces c-FOS phosphorylation on T232, the residue which controls its transcriptional activity, (Fig. 4), we questioned if there was a direct link between c-FOS and IAP expression. Specifically, since apoptosis is regulated by c-FOS [45,46] and IAP downregulation could potentially remove a blockade on apoptosis, we silenced c-FOS in MCF7-HER2 cells to determine whether c-FOS directly regulates the expression of IAPs. Silencing of c-FOS downregulated IAP protein expression compared to control conditions (Supplementary Fig.4), supporting the idea that the pro-apoptotic effects of Tetrocarcin-A could be mediated by down-regulation of c-FOS transcriptional activity.

3.5. Tetrocarcin-A targets primary breast cancer and lung cancer stem cells

To test the potential clinical relevance of our findings, we extended our studies to primary cultures derived from breast cancer surgical resections. Primary breast cells were more sensitive to the anti-growth effects of Tetrocarcin-A (Fig. 6a) than established cell lines (Fig. 1a and b). Downregulation of JAM-A was also evident in primary cells treated with Tetrocarcin-A (Fig. 6a). Of further interest was a population of cancer stem cells, since these cells have been proposed to play a pivotal role in treatment resistance and tumor relapse [47,48] and JAM-A has recently been described as an essential cancer stem cell maintenance factor [10,11]. LCSC-229 and -36 lung cancer stem cells derived from surgical specimens [24] expressed high levels of JAM-A, which was downregulated upon Tetrocarcin-A treatment (Fig. 6b). Tetrocarcin-A also significantly reduced growth in a concentration-dependent manner in these cells (Fig. 6b). In addition, Tetrocarcin-A triggered caspase activation in primary breast cancer and lung cancer stem cells (data not shown). Collectively the data suggest that Tetrocarcin-A inhibits the growth of cancer stem cells in conjunction with JAM-A downregulation, which in turn may inhibit the repopulating ability of cancer stem cells.

3.6. Tetrocarcin-A inhibits model tumor xenograft formation

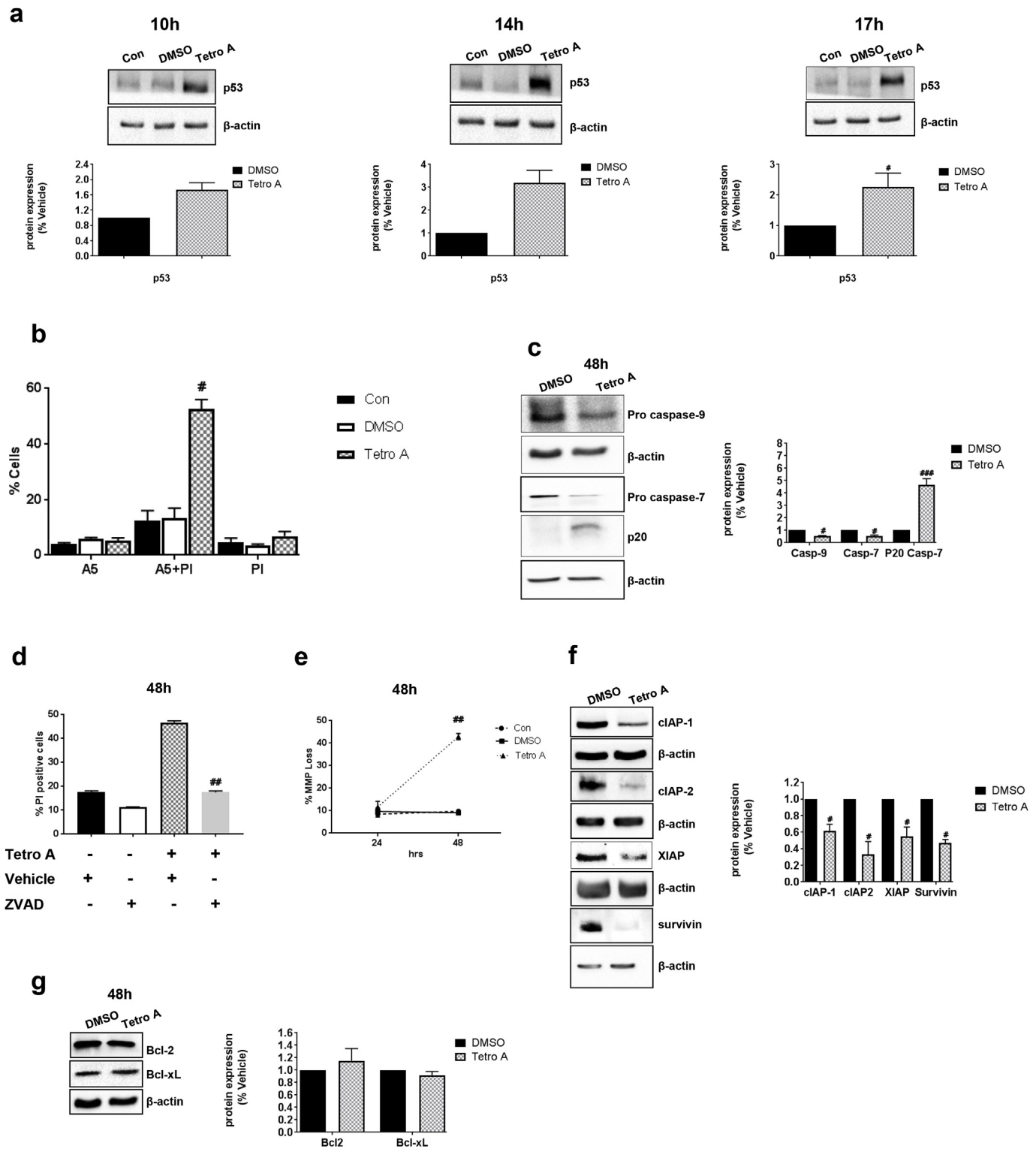
Finally, we studied the anti-tumor activity of Tetrocarcin-A using the *in ovo* or semi-*in vivo* chicken egg chorioallantoic membrane model, which has previously been validated for preclinical evaluation of anti-cancer agents [26,27]. Treatment of MCF7-HER2 xenografts with Tetrocarcin-A reduced gross size (Fig. 7a), induced widespread cell death, and reduced membranous expression of cytokeratin (CAM-5) and JAM-A (Fig. 7b). Although clusters of neoplastic cells were still observed infiltrating the CAM in Tetrocarcin-A-treated tumors, the proliferation index (calculated by the expression of Ki-67 in 500 tumor cells) was significantly reduced (Fig. 7c).

4. Discussion

Invasive spread of breast cancer cells to different organs means that metastasis is the main cause of death of breast cancer patients. Metastasis is a particular clinical risk when patients develop acquired resistance to targeted therapies, including some of the suite of kinase inhibitors currently used in precision medical oncology. One mechanism by which breast cancer cells could acquire invasive potential or become resistant to treatments is due to overexpression of proto-oncogenes or suppression of tumor suppressor genes. While not formally described as a proto-oncogene, overexpression of the tight junction protein JAM-A has been linked to aggressive disease and poor patient outcome in breast cancer [4]. Furthermore, JAM-A expression has been described as a novel regulator of HER2 protein degradation and signaling in breast cancer cells [3].

Since JAM-A abrogation or functional antagonism has been reported to stall tumor progression in mouse models of cancer [12,13] we screened a National Cancer Institute natural compound library using JAM-A overexpressing breast cancer cells to identify candidate JAM-targeting molecules. In the present study we show for the first time that the anti-tumor antibiotic Tetrocarcin-A exerts anti-growth effects on breast cancer cells at least in part by inducing lysosomal down-regulation of JAM-A protein expression, accompanied by reductions in HER2 and associated signaling effectors. Tetrocarcin-A also inhibited the growth of primary breast cancer cells and lung cancer stem cells. Our evidence suggests that the cytotoxic effects of Tetrocarcin-A involve inactivation of cell survival pathways and removal of a blockade on apoptosis, likely by suppressing the transcriptional activity of c-FOS (see model; Supplementary Fig.5).

MCF7-HER2 cells were principally used for our studies as they express very high levels of JAM-A, which in turn regulates the expression of HER2. However, the JAM-expressing cells SK-BR-3 (Supplementary Fig.3) and HCC38 human breast cancer cells in addition to 4T1 mouse mammary cancer cells were also found to be sensitive to the anti-growth effects of Tetrocarcin-A (data not shown). It is noteworthy that MCF7 cells are p53-wild type, whereas SK-BR-3 cells are p53-mutated [49], since mutation of p53 is a common mechanism whereby tumor cells can adapt to ensure their own survival and/or circumvent chemotherapeutic drug effects [50]. The fact that Tetrocarcin-A reduced



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the growth of p53-mutant as well as p53-wild type cells is an encouraging indicator of potential value in real cancer settings.

To improve stringency in the cell growth assays, cells were only exposed to Tetrocarcin-A after a population doubling, since cancer cells with well-established cell contacts have higher resistance to chemotherapeutic treatments [51]. Tetrocarcin-A still reduced growth in a concentration- and time-dependent manner in all cells tested. Encouragingly, Tetrocarcin-A also exerted anti-growth effects on JAM-A-expressing BT474 cells that had been conditioned to become resistant to the HER2-targeted therapy Trastuzumab [20,21]; its bioactivity in fact exceeding that in drug-sensitive BT474 cells. Since cancer therapies are frequently combined, it was also encouraging to note that sublethal concentrations of Tetrocarcin-A and cisplatin in combination with each other exerted synergistic anti-growth effects on MCF7-HER2 breast cancer cells, and suppressed the clonogenic survival of breast cancer cells. It cannot be ignored, however, that normal-like epithelial cells

Fig. 5. Tetrocarcin-A induces caspase-dependent apoptosis in breast cancer cells. (a) MCF7-HER2 cells were seeded in 6-well plates and treated at their doubling time with 2.5 μM Tetrocarcin-A (The NCI/DTP Open Chemical Repository) or vehicle (0.01% v/v DMSO). Cell extracts were prepared at 10, 14 and 17h and western blotting performed for human p53. (b) MCF7-HER2 cells were plated in 6-well plates and treated at their doubling times with 2.5 μM Tetrocarcin-A or vehicle (0.01% v/v DMSO). After 48h treatment, cells were stained using an Annexin V/propidium iodide (PI) kit and images acquired on a BD FACS-Calibur flow cytometer. (c) MCF7-HER2 cells on 6-well plates were treated at their doubling time with 2.5 μM Tetrocarcin-A or vehicle (0.01% v/v DMSO). Cell extracts were prepared at 48h and western blotting performed for human caspase-7, caspase-9, p20 caspase-7 and β-actin. (d) MCF7-HER2 cells were plated in 24-well plates and treated at their doubling time with 2.5 μM Tetrocarcin-A or vehicle (0.01% v/v DMSO). After 1h incubation, cells were treated for 48h with the caspase inhibitor ZVAD-FMK (50 μM) or vehicle control (0.01% v/v DMSO). Cells were stained with PI as per manufacturer's instructions, and images acquired on a BD FACS-Calibur flow cytometer. (e) MCF7-HER2 cells were plated in 24-well plates and treated at their doubling time with 2.5 μM Tetrocarcin-A or vehicle control (0.01% v/v DMSO). After 48h treatment, cells were stained with TMRM and images acquired on a BD FACS-Calibur flow cytometer. (f,g) MCF7-HER2 cells were treated at their doubling time with 2.5 μM Tetrocarcin-A or vehicle (0.01% v/v DMSO). Cell extracts were prepared at 48h, separated by SDS polyacrylamide gel electrophoresis and immunoblotted for human cIAP-1, XIAP, cIAP-2, survivin, Bcl-2, Bcl-xL or β-actin. Error bars refer to standard error of the mean of triplicate experiments (#p < 0.05; ###p < 0.005; ## p < 0.00001, by two-tailed unpaired student's *t*-test. Comparisons were made between vehicle controls vs Tetrocarcin-A. All experiments were independently repeated three times, and representative blots shown.

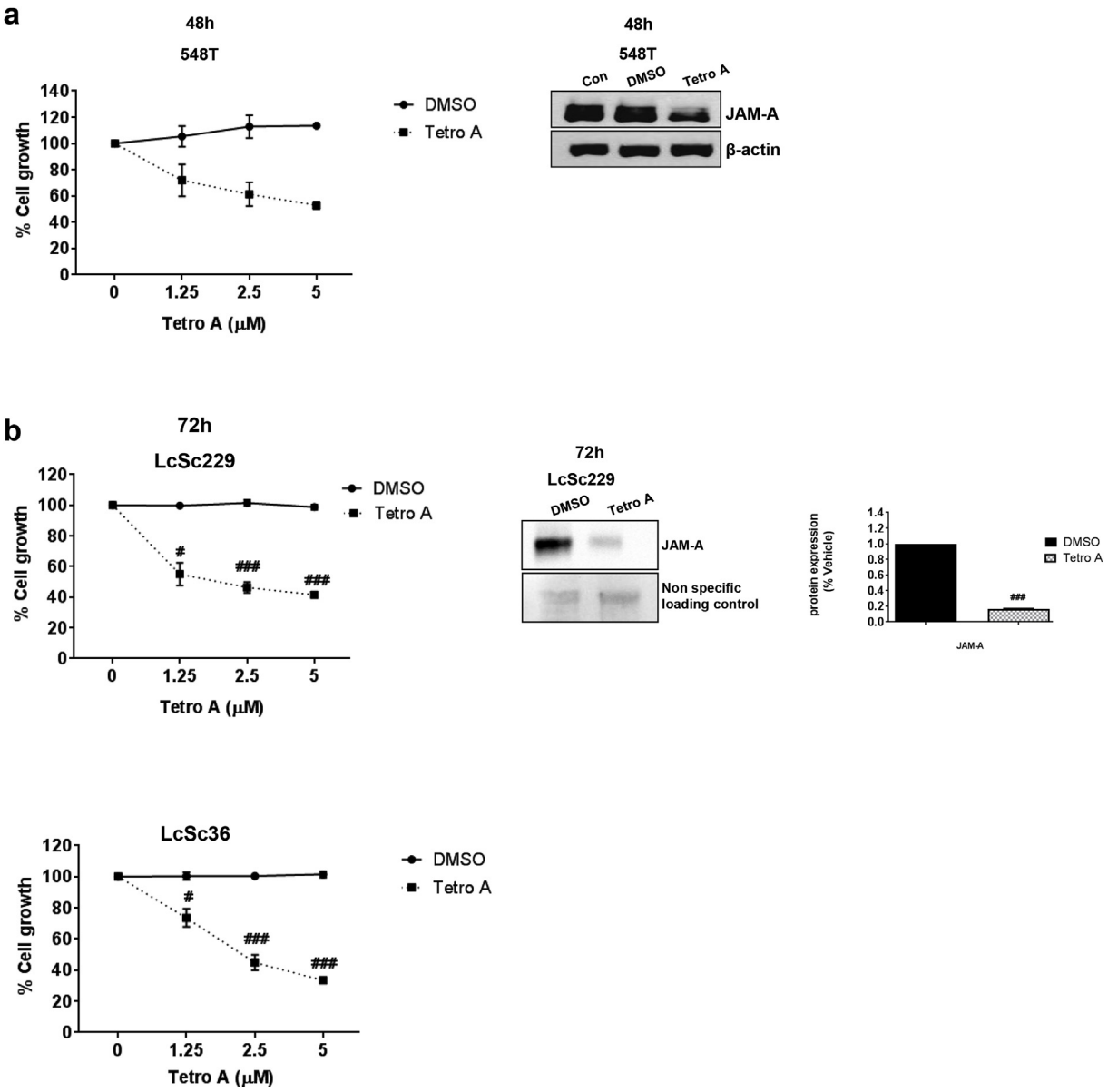

Fig. 6. Tetrocarcin-A targets patient-derived primary breast cancer cells and lung cancer stem cells. Primary breast cancer cells (a) were isolated as described [23] and cultured in Mammary Epithelial Cell Growth Medium. Lung cancer stem cells (b) were isolated and cultured as previously described [24]. Cells were plated in 96-well plates or 6-well plates and treated with the indicated concentrations of Tetrocarcin-A or vehicle (0.006–0.02% v/v DMSO). Cell growth was assessed by Alamar Blue assay at 48 or 72h, or cell extracts were prepared at 48 or 72h, separated by SDS polyacrylamide gel electrophoresis and immunoblotted with antibodies to human JAM-Aor β-actin. Error bars refer to standard error of the mean of triplicate experiments (#p < 0.05; by two-tailed unpaired student's *t*-test. Comparisons were made between vehicle controls vs Tetrocarcin-A). Experiments with immortalized cells were repeated three times and representative blots shown. The blots for primary breast cancer cells were done once.

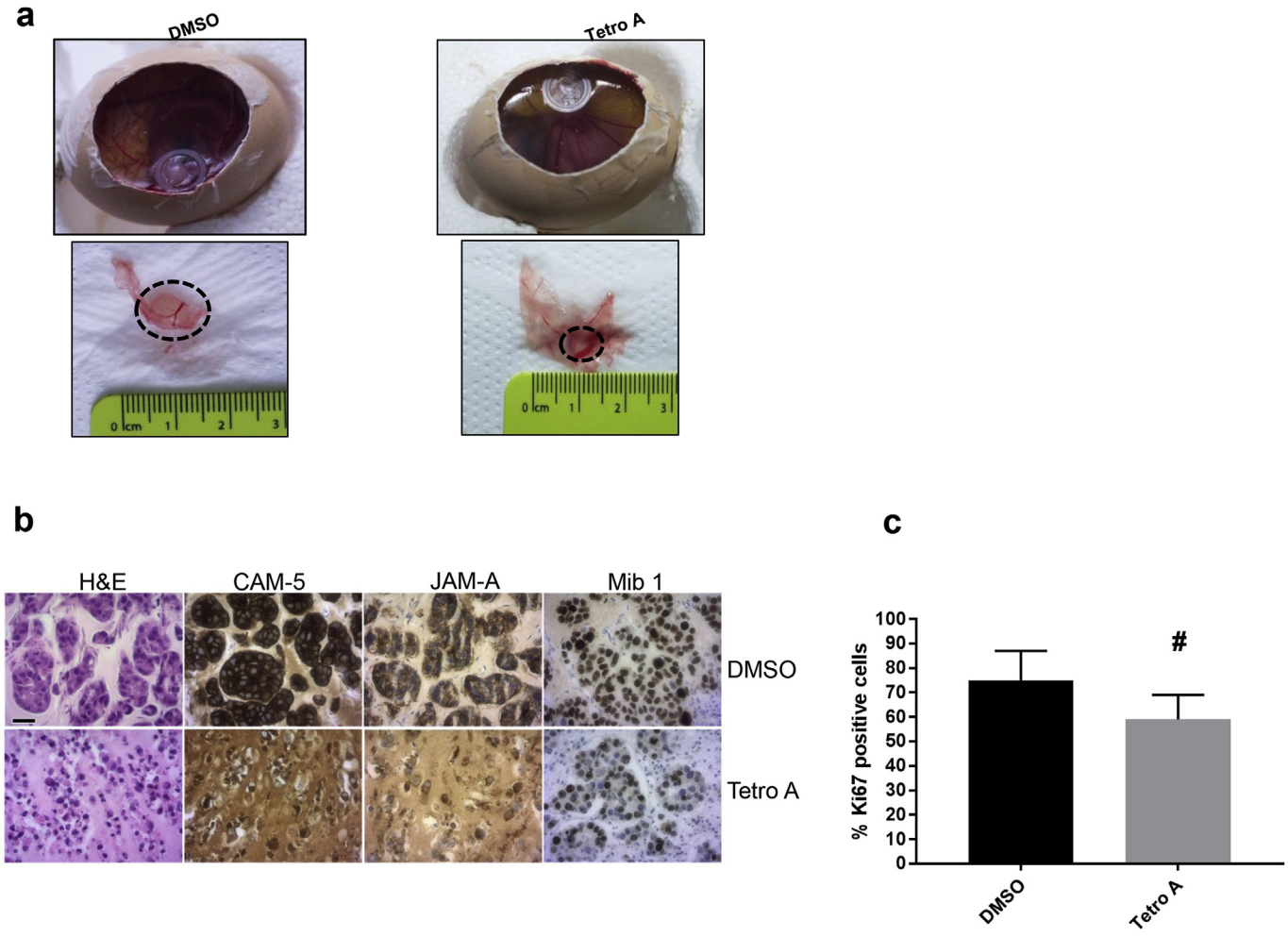

Fig. 7. Tetrocarcin-A inhibits the growth of breast cancer cells in a semi-*in vivo* chorioallantoic membrane xenograft model. *In vivo* chorioallantoic membrane assays were performed as described previously [26,27]. Briefly, 2×10^6 MCF7-HER2 tumor cells were resuspended in 25 µL serum-free medium and 25 µL Matrigel and implanted on the chorioallantoic membrane of fertilized chicken eggs on day 8 of gestation. Tumors were topically treated with 2.5 µM Tetrocarcin-A in 15 µl PBS or vehicle (0.01% v/v DMSO in 15 µl PBS) from days 10–13 inclusive. Tumors and their surrounding membrane were sampled on day 13, fixed in 4% (w/v) paraformaldehyde, paraffin-embedded and cut into 5 µm sections. Sections were deparaffinised in xylene and stained for anti-human JAM-A, Ki67 (Mib1) and cytokeratin (CAM-5). Representative gross (a) and microscopic (b) images (scale bar, 25 µm) are shown, in addition to the percentage of cells negative for Ki67 based on 500 cells in the intact tumor islands adjacent to the membrane (c). Error bars refer to standard error of the mean of triplicate experiments (#$p < 0.05$; ###$p < 0.005$, by two-tailed unpaired student's *t*-test; with comparisons made between vehicle controls vs Tetroarcin-A-treated tumors). The assay was repeated 3 times.

were even more sensitive to the anti-growth effects of Tetrocarcin-A than MCF7-HER2 cells. This is not surprising in the cell line we tested, MCF 10A, as these cells express high levels of JAM-A [3]. Nonetheless, it illustrates the point that “bystander damage” is a frequent side-effect of chemotherapeutic drugs; and highlights the value of ongoing research into combinatorial approaches towards targeted drug delivery to neoplastic rather than normal cells.

In investigating the mechanism of action of Tetrocarcin-A, we discovered a partial role for lysosomally-induced degradation of JAM-A in conjunction with that of HER2. Furthermore, supportive evidence that JAM-A degradation likely precedes that of HER2 was obtained from genetic silencing studies, in which JAM-A silencing was found to reduce the protein levels of HER2, without reciprocicity of effect [3]. Typically HER2 has been shown to be degraded by a proteosomal rather than lyosomal pathway [52], however recent evidence supports the possibility that anti-HER2 therapies could in fact promote HER2 degradation by lysosomes [53]. However junctional proteins like JAM-A would be expected to be degraded by lysosomal rather than proteasomal mechanisms. We cannot exclude the possibility that HER2 degradation is still proteasomal, and merely induced secondary to JAM-A loss in the lysosome. Regardless, the partial rescue of JAM-A and HER2 expression in Tetrocarcin-A-treated cells pre-exposed to a lysosomal inhibitor, in addition to the partially protective effect of JAM-A overexpression, support the likelihood of a central role for JAM-A in the mechanism of action of Tetrocarcin-A. This partial rescue could be significant in the clinical setting of breast tumors that express high levels of JAM-A.

Downstream of JAM-A, the loss of several cell survival signaling molecules could account for compromised cell growth following Tetrocarcin-A treatment. One such cell survival pathway which we and others have been shown to be inhibited upon JAM-A silencing is AKT, specifically via reduced phosphorylation at serine-473 [3,8]. However recent studies have shown phosphorylation of Thr-308 is a more reliable biomarker for the protein kinase activity of AKT in tumour samples than Ser-473 [54]. In this study, Tetrocarcin-A inhibited phosphorylation of AKT on both serine-473 and threonine-308 downstream of JAM-A loss. In addition to AKT, we and others have also found that overexpression of JAM-A upregulates ERK1/2 phosphorylation (data not shown, [32]), while in our study gene silencing of JAM-A in MCF7 HER2 breast cancer cells downregulated ERK1/2 phosphorylation. Similar downregulation of ERK1/2 phosphorylation was observed in Tetrocarcin-A-treated cells, suggesting that Tetrocarcin-A cytotoxicity is associated with phospho-ERK1/2 downregulation secondary to JAM-

A loss.

Inhibition of AKT or ERK1/2 phosphorylation causes apoptosis, and accordingly our data showed that Tetrocarcin-A induces caspase-dependent apoptosis in breast cancer cells. We speculate that this involves c-FOS downstream of the ERK pathway, since c-FOS is transcriptionally activated upon phosphorylation of T-232 by ERK [35,36] and Tetrocarcin-A treatment or JAM-A silencing similarly reduced c-FOS phosphorylation on this residue. The same conditions inhibited nuclear localization of c-FOS. We also examined the expression of anti-apoptotic proteins downstream of Tetrocarcin-A treatment, and noted downregulation of several IAPs in addition to survivin. Since overexpression of IAPs contributes to the progression of solid and hematological tumors [55–57], and several IAP-targeting drugs are in clinical trials [58,59], it is possible that Tetrocarcin-A may have pharmacological value in this setting. Our data therefore suggest the following model. Tetrocarcin-A, working at least in part by reducing JAM-A expression, exerts a negative tone on the phosphorylation and nuclear localization of c-FOS. This in turn reduces the expression of targets such as the inhibitor of apoptosis proteins (IAPs), which de-inhibits apoptosis and sends cells into a death pathway.

Despite promising indications that JAM-A loss downstream of Tetrocarcin-A treatment reduced the growth of terminally-differentiated breast cancer cells, several studies now propose cancer stem cells to be responsible for disease relapse and resistance to clinical therapeutic compounds [60,61]. However, since JAM-A has been described to be required for maintenance of cancer stem cells in various settings including glioblastoma [62], we reasoned that Tetrocarcin-A might be effective against JAM-high cancer stem cells. As expected, Tetrocarcin-A inhibited the growth of lung cancer stem cells in conjunction with downregulating JAM-A. Together with the ability of Tetrocarcin-A to inhibit tumor growth in an *in ovo*/semi-*in vivo* model, this supports the value of further investigations centering upon Tetrocarcin-A as a novel therapeutic compound. Although *in vivo* mouse model studies would be necessary further along the route to regulatory approval, it is worth noting that the *in ovo*/semi-*in vivo* setting of the chick embryo chorioallantoic membrane model has been widely used as an option for testing cancer drugs [63,64], and may lend itself to fast and cost-effective patient-derived xenograft studies in the future.

In conclusion, we have shown for the first time that the anti-tumor antibiotic Tetrocarcin-A is cytotoxic to breast cancer cells following JAM-A and HER2 downregulation via a pathway involving lysosomes. The fact that drug-induced cytotoxicity was attenuated in JAM-overexpressing cells supports a key role for JAM-A in the mechanism of action of the compound; as does our observation that HER2 expression levels are sensitive to those of JAM-A (but not the other way around). We have also shown for the first time that Tetrocarcin-A reduces both the nuclear levels of c-FOS and levels of T-232-phosphorylated c-FOS, which would restrain its transcriptional activity. These events were associated with downregulation of IAPs and removal of their blockade on caspase-dependent apoptosis. Importantly, recent evidence suggests that JAM-A is required for the repopulating ability and maintenance of cancer stem cells [10,11], and here we show Tetrocarcin-A-induced reductions in JAM-A expression alongside cell growth inhibition in cancer stem cells. Since current anti-cancer drugs typically target rapidly-dividing populations of terminally-differentiated cells rather than slowly-dividing cancer stem cells [65,66], these data suggest promise for pharmacological JAM-A targeting as a novel strategy in certain cancers. This is supported by pre-clinical data indicating that Tetrocarcin-A exerts anti-tumorigenic effects in a semi-*in vivo* model. Although molecules in addition to JAM-A may play a role in this phenomenon, the emerging role of JAM-A as a master regulator of the expression of cancer-relevant proteins will make for intriguing future avenues of research.

Conflicts of interest

The authors have no conflicts of interest to declare.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.09.032.

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