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Corresponding Author: Prof. Vassilis Zoumpourlis, PhD

Corresponding Author's Institution: National Hellenic Research Foundation

First Author: Stella Logotheti, PhD

Order of Authors: Stella Logotheti, PhD; Nikolas Khoury; Spiros A Vlahopoulos, PhD; Elena Skourti, MSc; Dimitra Papaevangeliou, PhD; Triantafyllos Liloglou, PhD; Vassilis Gorgoulis, MD, PhD; Irina Budunova, PhD; Anthony M Kyriakopoulos, MD, PhD; Vassilis Zoumpourlis, PhD

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Abstract: Glucocorticoids (GCs) are frequently used in anticancer combination regimens; however, their continuous use adds selective pressure on cancer cells to develop GC-resistance via impairment of the glucocorticoid receptor (GR), therefore creating a need for GC-alternatives. Based on the drug repurposing approach and the commonalities between inflammation and neoplasia, drugs that are either in late-stage clinical trials and/or already marketed for GC-refractory inflammatory diseases, could be evaluated as GC-substitutes in the context of cancer. Advantageously, unlike new molecular entities currently being de novo developed to restore GC-responsiveness of cancer cells, such drugs have documented safety and efficacy profile, which overall simplifies their introduction in clinical cancer trials. In this study, we estimated the potential of a well-established, multistage, cell line-based, mouse skin carcinogenesis model to be exploited as an initial screening tool for unveiling covert GC-substitutes. First, we categorized the cell lines of this model to GC-sensitive and GC-resistant, in correlation with their corresponding GR status, localization and functionality. We found that GC-resistance starts in papilloma stages, due to a dysfunctional GR, which is overexpressed, DNA binding-competent, but transactivation-incompetent in papilloma, squamous and spindle stages of the model. Then, aided by this tool, we evaluated the ability of N-bromotaurine, a naturally-occurring, small-molecule, NSAID which is under consideration for use interchangeably/in replacement to GCs in skin inflammations, to restore antiproliferative response of GC-resistant cancer cells. Unlike GCs, N-bromotaurine inhibited cell-cycle progression in GC-resistant cancer cells and efficiently synergized with cisplatin, thus indicating a potential to be exploited instead of GCs against cancer.

N-bromotaurine surrogates for loss of antiproliferative response and enhances cisplatin efficacy in cancer cells with impaired glucocorticoid receptor

Stella Logotheti[1,7], Nikolas Khoury[1], Spiros A. Vlahopoulos[2], Elena Skourti[1], Dimitra Papaevangeliou[1], Triantafyllos Liloglou[3], Vassilis Gorgoulis[4], Irina Budunova[5], Anthony M. Kyriakopoulos[6], Vassilis Zoumpourlis[1*]

[1]Biomedical Applications Unit, Institute of Biology, Medicinal Chemistry and Biotechnology, National Hellenic Research Foundation, 48 Vassileos Constantinou Avenue, 116 35 Athens, Greece.
[2]Horemio Research Institute, First Department of Pediatrics, "Aghia Sophia" Children's Hospital, National and Kapodistrian University of Athens, Athens, Greece.
[3]University of Liverpool, Department of Molecular and Clinical Cancer Medicine, 200 London Road, Liverpool L3 9TA, UK
[4]Laboratory of Histology-Embryology, Molecular Carcinogenesis Group, Medical School, National and Kapodistrian University of Athens, Athens, Greece.
[5]Department of Dermatology, Northwestern University, Chicago, IL, USA.
[6]Nasco AD, Biotechnology Laboratory, 11th Sachtouri str., Piraeus, Greece.
[7]Department of Biotechnology, Agricultural University of Athens, Athens, Greece

* Corresponding author
Tel: 30-10-7273730
Fax: 30-10-7273677
e-mail: vzub@eie.gr

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Keywords: GR; glucocorticoid resistance; N-bromotaurine; skin carcinogenesis; drug re-purposing

Abbreviations: GC: glucocorticoid; GR: glucocorticoid receptor; GRE: glucocorticoid receptor element; NSAID: non-steroid anti-inflammatory drug; BrTaur: N-bromotaurine; ClTaur: N-chlorotaurine; EMSA: electrophoretic mobility shift assay; FACS: fluorescence-activated cell sorting; qRT-PCR: quantitative Real-Time-Polymerase Chain Reaction.

ABSTRACT

Glucocorticoids (GCs) are frequently used in anticancer combination regimens; however, their continuous use adds selective pressure on cancer cells to develop GC-resistance via impairment of the glucocorticoid receptor (GR), therefore creating a need for GC-alternatives. Based on the drug repurposing approach and the commonalities between inflammation and neoplasia, drugs that are either in late-stage clinical trials and/or already marketed for GC-refractory inflammatory diseases, could be evaluated as GC-substitutes in the context of cancer. Advantageously, unlike new molecular entities currently being *de novo* developed to restore GC-responsiveness of cancer cells, such drugs have documented safety and efficacy profile, which overall simplifies their introduction in clinical cancer trials. In this study, we estimated the potential of a well-established, multistage, cell line-based, mouse skin carcinogenesis model to be exploited as an initial screening tool for unveiling covert GC-substitutes. First, we categorized the cell lines of this model to GC-sensitive and GC-resistant, in correlation with their corresponding GR status, localization and functionality. We found that GC-resistance starts in papilloma stages, due to a dysfunctional GR, which is overexpressed, DNA binding-competent, but transactivation-incompetent in papilloma, squamous and spindle stages of the model. Then, aided by this tool, we evaluated the ability of N-bromotaurine, a naturally-occurring, small-molecule, NSAID which is under consideration for use interchangeably/in replacement to GCs in skin inflammations, to restore antiproliferative response of GC-resistant cancer cells. Unlike GCs, N-bromotaurine inhibited cell-cycle progression in GC-resistant cancer cells and efficiently synergized with cisplatin, thus indicating a potential to be exploited instead of GCs against cancer.

INTRODUCTION

Glucocorticoids (GCs) are steroid hormones which inhibit tumor cell proliferation, mitigate chemotherapy side effects and enhance efficiency of anticancer agents[1,2]. They are frequently included in combination anticancer therapies, either as palliative agents against chemotherapy-induced-nausea-and-vomiting or as antiproliferative agents. Their effects are mediated by the glucocorticoid receptor (GR). GC binds to GR which, following dissociation from a cytoplasmic chaperone/co-chaperone complex, translocates to the nucleus, homodimerizes and regulates gene expression. GR transactivates or transrepresses genes by direct binding to GREs (glucocorticoid responsive elements), by tethering itself to other transcription factors apart from DNA binding, or in a composite manner by both direct GRE binding and interactions with transcription factors bound to neighboring sites (reviewed by Ramamoorthy et al[2]). Thus, a functional GR elicits tumor-suppressive events in a pleiotropic manner, through a plethora of mechanisms and crucial pathways.[3-5]

Theoretically, the clinical benefits demonstrated by the long-term experience on GCs, combined with the tumor-suppressive nature of GR, offer a strong alibi for their routine use in cancer therapeutics (e.g. prostate cancer, breast cancer, leukemia), as evidenced by the increasing number of GC-containing combination regimens, both established and investigational. Nevertheless, GC cotreatment often induces resistance towards cancer therapy[1,6] thus raising concerns regarding the tendency of GC-sensitive cancer cells to develop resistance upon frequent GC use. Mechanistically, this tendency is attributed to the pleiotropic nature of GR *per se*. GR's pleiotropy becomes a double-edged sword, since cancer cells have, at their disposal, as many potentials to overcome GR's antiproliferative barrier and achieve GC-resistance, as is the plethora of underlying GR-mediated antiproliferative pathways they can defuse one way or another. For neutralizing GR antiproliferative effects, cancer cells exploit several strategies, which in several cases simultaneously co-exist in a cancer cell (e.g. reduced GR expression, reduced DNA binding ability, GR mutations and polymorphisms, co-expression of dominant negative GR isoforms, impairment of transrepression mode et.c.).[2,7,8] GC-unresponsiveness by GR impairment may start as early as the benign stages, highlighting that inactivation of GC/GR axis is a selective advantage in order for a cancer cell to surpass the antiproliferative break and continue its tumorigenic march towards aggressive stages.[9]

This overall necessitates alternatives restoring and/or surrogating for GCs' antiproliferative effects.[2,10] To this end, innovative synthetic molecules are being developed by academia, aiming to re-activate GR-mediated antiproliferative pathways and restore GC-responsiveness of cancer cells[10]. If they successfully pass clinical trials, they will eventually find their way to the bedside. But this established bench-to-bedside pipeline is not an one-way street. The recently framed concept of drug repurposing suggests evaluating suitability of

known drugs for use in new indications[11]. Implementing this concept in the issue of GC-resistance, we postulate that drugs which are already in late stages of clinical trials or approved for indications other than cancer may be latent GC-substitutes, able to imitate aspects of the GC therapeutic profile. These hypothetical latent GC-substitutes might be competent to either restore the antiproliferative phenotypes in GC-resistant cells; or to be used interchangeably to GCs, in order to reduce the selective pressure exerted by continuous use of GCs on GC-sensitive cells, thus preventing/delaying their clonal expansion to GR-impaired and, hence, GC-resistant cells. A clinical advantage of the proposed approach is that these candidate substances are actually closer to the bedside than they are to the bench, since they have a more characterized efficacy and safety profile, in terms of their documentation for other therapeutic indications. This is translated to both faster filing and regulatory approval procedures and to reduced financial costs to develop these substances as anticancer agents, compared to starting the anticancer drug research-and-development workflow from scratch, i.e. by developing New Molecular Entities (NMAs, defined by FDA as experimental substances without precedent among regulated and approved drug products).[11]

Except for anticancer agents, GCs are also common anti-inflammatory agents. In fact, their use against inflammations historically preceded their use against neoplasias. GC-resistance is a frequent problem in inflammations as well, and drugs are being developed in replacement of GC-containing anti-inflammatory regimens.[12] Emerging GC-substitutes that are currently in the investigational clinical setting for inflammation management include, but are not limited to, the NSAID (Non-Steroid Anti-Inflammatory Drug) taurine haloamine derivatives, mainly N-bromotaurine (BrTaur) and N-chlorotaurine (ClTaur). These are generated by eosinophils and neutrophils at a site of inflammation and exert potent anti-inflammatory properties. Other common features shared with GCs is their naturally-occurring and small-molecule nature and their immunomodulatory and antimicrobial properties[13]. Another common characteristic is the potential to ameliorate chemotherapy-induced nausea and vomiting, since their maternal substance, i.e. the non-essential aminoacid taurine, which is orally administered as a pro-drug in order to be converted to the haloamine derivative at the site of inflammation, was recently proven clinically capable of such an effect in leukemic patients.[14] Advantageously, taurine haloamines have shown good efficacy, tolerance and insignificant toxic effects upon topical use on clinical patients who are refractory to conventional GC-based anti-inflammatory therapies[13,1516]. Based on the association between chronic inflammatory diseases and neoplasias,[17,18] we postulated that a drug which exerts overlapping features and common therapeutic indications with GCs and is able to surrogate for GCs in GC-resistant inflammations may be able to surrogate for the GCs' antiproliferative function in GC-resistant cancer cells as well.

As a springboard for testing this hypothesis, we took advantage of our long-term experience on a well-established mouse model of skin carcinogenesis.[19] This comprises of a series of cell lines which represent different stages of mouse skin tumor progression and are categorized on the basis of increasing aggressiveness to immortalized keratinocytes (C5N), benign papillomas (P6), malignant squamous carcinomas (B9), and highly invasive spindle cells (A5, CarB).[19] The B9:A5 pair represents the clonal expansion from squamous to spindle stages. The model has been developed in Dr. A. Balmain's lab and has been thoroughly reviewed[18] and described previously.[20] Briefly, in order to obtain these cell lines, each of which represent the initiation, promotion or progression stages of skin carcinogenesis, a chemical carcinogenesis protocol on mice was applied. The normal epidermis of mice or normal epithelial mouse cells were treated with a single dose of the polycyclic aromatic hydrocarbon 7,12 dimethylbenz[a]anthracene (DMBA), followed by weekly applications of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). This led to the development of numerous benign papillomas, some of which progressed to malignant squamous cell carcinomas several weeks after the first exposure to carcinogens and cell lines were produced from these tumors. Overall, this model poses the following advantages: a) it is multistage, meaning that it simulates the step-wise manner by which a tumor initiates, promotes and progresses, b) it is coherent, since cell lines have been derived in a consistent manner following a meticulous chemical carcinogenesis protocol, c) although skin-tissue based, it further applies to almost all epithelial cancers.[21] Furthermore, the fact that the model is skin tissue-based facilitates our analysis, because skin cancer is a traditional field where the mechanisms of GR function in correlation to GC-responsiveness have been adequately studied.[4,5,9] Therefore, although our model has never been characterized before in terms of GC-responsiveness, it stood a good chance to faithfully mirror or even complement previous robust findings.

First, we characterized our system in terms of antiproliferative response to GCs. Then, we correlated this responsiveness to the underlying GR expression status, localization and functionality. Finally, we checked the ability of BrTaur to bypass GC-resistance of cancer cells either alone or in combination with cisplatin.

MATERIALS AND METHODS

Cells and culture conditions

Mouse cell lines of the mouse skin carcinogenesis model have been produced and obtained by Dr. Allan Balmain. All human cell lines used were obtained by American Type Culture Collection (ATCC). Cells were cultured as previously described.[22] The preparation[23] and use

of BrTaur is covered by licensing agreement. The in-house formulation was donated by NASCO AD Biotechnology Laboratory for preclinical research purposes.

Proliferation assays

Collectively, 2250 cells per well were seeded in 96-well plates. After cells were attached, the first measurement was taken. This time point is called 0 hours. At this point, 24h after seeding, dexamethasone (10^{-9} – 10^{-6}M, from a stock of 10^{-3}M dexamethasone diluted in ethanol), BrTaur (25μM-250μM from a stock of 4mM BrTaur) or taurine (5-50mM from a stock of 200mM taurine diluted in water) was added and measurements were taken after 24, 48 and 72h. For co-treatment experiments, cells were treated with either 10^{-7}M dexamethasone or BrTaur (125, 250 or 500μM) 24-hours prior, con-currently with or 24-hours post cisplatin treatment. Untreated cells were used as controls. Following treatments, cells were fixed with 100% methanol and, then, crystal violet solution was added to each well. After a 10-minute incubation in room temperature, each well was washed 3 times with 200μL water, and plates were incubated on a shaker for 45min. Optical Density (OD) was measured at 595nm using a Tecan reader. The data was transferred to Microsoft Excel and analyzed. Background absorbance was corrected using triplicate sets of wells containing medium only (no cells) and crystal violet reagent as per experimental well. Three independent experiments were performed and each one of them included a triplicate value set.

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RNA extraction, cDNA synthesis and qRT-PCR analysis

mRNA extraction, cDNA synthesis and quantitative real-time PCR was conducted as previously described.[24] Primers used appear in Table I.

Preparation of cell lysates and Western blot analysis

Total, cytoplasmic and nuclear cell lysates were prepared as previously described.[25] The primary antibody was an in-house anti-GR rabbit polyclonal antibody, clone 2F8, against aminoacids 305-427 of the N-terminal domain (kindly provided by Dr. M.N. Alexis), in a 1:500 dilution. Primary anti-beta-actin antibody in a 1:1000 dilution was used as a loading control. The secondary antibody was a mouse anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in a 1:1000 dilution.

Two-stage chemical carcinogenesis protocol

Tumors induced on mouse skin following a chemical carcinogenesis protocol were fixed and paraffin-embedded. Slides carrying formalin-fixed paraffin-embedded (FFPE) mouse skin papilloma and squamous and spindle tumors were prepared as previously described.[22] In

vivo experiments were performed in the in-house authorized animal house. Experiments complied with the Protocol on the Protection and Welfare of Animals, as obliged by the rules of the National Hellenic Research Foundation, the regulations of the National Bioethics Committee and the article 3 of the presidential decree 160/1991 (in line with 86/609/EEC directive) regarding the welfare of experimental animals.

Immunohistochemical and immunocytochemical staining

Immunohistochemical staining on FFPE sections was performed as described earlier.[26] The sections were stained with anti-GR, clone 2F8, in a 1:10 dilution. For the immunocytochemistry staining with anti-GR antibody 2F8, we followed the same procedure, incubation periods and reagents, by omitting the deparaffinization step.

Immunofluorescence staining

Cells were grown and fixed on coverslips and where subjected to immunofluorescence staining as previously described.[27] The slides were incubated with primary antibody anti-GR, clone M-20 (Santa Cruz Biotechnology, Santa Cruz, CA) in a 1:50 dilution. The secondary antibody was anti-rabbit FITC-conjugated (Jackson Laboratory, Bar Harbor, Maine, USA) diluted in 1:100.

Electrophoretic Mobility Shift assay

Annealed oligonucleotides for the *human metallothionin IIA* Glucocorticoid Responsive Element (5'-TGGTACACTGTGTCCTGAATTCA-3' and 5'-TGAATTCAGGACACAGTGTACCA-3') were end-labeled with γ^{32}P-ATP using T4-polynucleotide kinase and the reaction products were purified on a 8% polyacrylamide gel. DNA binding reactions were performed as previously described.[28] For the supershift control experiment, the primary polyclonal antibody anti-GR 10-10 (kindly provided by Dr. M. Alexis) was used.

Plasmids, transfections and luciferase reporter assay

A luciferase plasmid carrying GRE sequences (17m-GRE-G-Luc), as well as a control vector carrying no GRE binding site (tata-pG13Luc) described previously[29] were used for transfections. Where indicated, cells were incubated with dexamethasone and transfected with by the calcium phosphate method, as described previously[7]. The luciferase activity was measured using a luminometer and was normalized for transfection efficiency with the β-galactosidase activity.

FACS analysis

Cells were harvested, trypsinized and centrifuged at 1,000rpm for 5min, at room temperature. The pellet was resuspended in 500μL PBS, fixed with 80% ethanol, vortexed, and stained with propidium iodide (50μg/mL), in the presence of 5mmol/L $MgCl_2$ and 10μg/mL RNase A in 10mmol/L Tris-HCl (pH 7.5). DNA content was analyzed on a FACSCalibur (Becton Dickinson) using the Modfit software.

Statistical analysis

Data are expressed as mean±SD. Each experiment was performed in triplicates. Then, the triplicate set values of three independent experiments were analyzed. For statistical analyses of proliferation assays results~~experiments~~, ~~,~~ANOVA utilising Dunetts' T3 post-hoc analysis ~~the student's t-test~~ was applied. QPCR results were evaluated using Mann-Whitney's test. Luciferase assays were analyzed using independent student's t-tests. P values of less than 0.05 were considered significant.

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RESULTS

The antiproliferative effect of GCs is lost in the promotion and progression stages of mouse skin carcinogenesis

Our first priority was to explore the GC antiproliferative effect on the cell lines of our system. GC effect ranges from proliferative in very low concentrations, to cytostatic/antiproliferative in more physiological concentrations and cytotoxic/apoptotic in higher concentrations.[30] To monitor GC effect on our system, we treated cells with a range of dexamethasone concentrations previously demonstrated to show antiproliferative effects on mouse keratinocytes (10^{-9}M - 10^{-6}M),[9] and subjected them to proliferation assay. Consistent with previous similar findings,[9] only the immortalized C5N cells were growth-inhibited by dexamethasone, in a concentration-dependent manner. P6, B9, A5 and CarB cells continued to proliferate despite dexamethasone presence (Fig. 1a). Dexamethasone induced no effect in P6, B9, A5 and CarB cells, neither proliferative nor antiproliferative. On the other hand, each tested dexamethasone concentration reduced proliferation rate of the GC-responsive C5N cells in a time-dependent manner (Fig. 1b). ANOVA utilising Dunetts' T3 post-hoc analysis demonstrated that C5N cells showed significant sensitivity to dexamethasone. In particular, at 72 hours, significant loss of survival was observed at 10^{-8}, 10^{-7}, and 10^{-6} concentrations ($p=0.014$, $p=0.002$, $p<0.001$ respectively). On the contrary, no significant difference observed between the untreated and treated P6 cells at any dexamethasone concentration (Fig. 1b). Therefore, the cells of our system were categorized to GC-sensitive (C5N; susceptible to growth inhibition by GCs) and to GC-resistant (P6, B9, A5 and CarB; no response to GCs, neither proliferative nor antiproliferative).

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GR expression and localization status in the multistage mouse skin carcinogenesis model

Then, we monitored GR expression and localization in each cell line of our model. First, western blot revealed an elevation of total GR levels towards more aggressive cancer stages. Nuclear GR levels gradually increase, showing an abrupt increase during B9-to-A5 transition, whereas there is a reduction of cytoplasmic GR protein levels from B9 to A5 cells, documenting a switch of the cytoplasmic-to-nuclear ratio upon B9/A5 transition, which is independent from GC presence (Fig. 2b). This tendency of GR to translocate to the nucleus during squamous-to-spindle transition was further confirmed by immunocytochemistry, which revealed cytoplasmic GR localization in C5N, P6 and B9 cells, and mixed cytoplasmic-nuclear localization in A5 and CarB cells (Fig. 2c). The GR nucleocytoplasmic translocation at the squamous-to-spindle threshold was additionally confirmed by immunofluorescence in B9 and A5 cells, which revealed a clear cytoplasmic signal in B9 cells, but an intense nuclear staining in A5 cells (Fig. 2d). To the best of our knowledge, this mixed cytoplasmic and nuclear GR localization in aggressive stages has never been reported before. To exclude the possibility that this observation is a cell-line artefact, we confirmed it immunohistochemically *in vivo*, on sections from skin tumors induced in mice following a chemical carcinogenesis protocol. Indeed, on tumors of the same animal, papilloma stage presents mainly cytoplasmic GR localization whereas in the corresponding squamous stage GR localization is more intense and gets even more intense in the spindle stage-tumors (Fig. 2e).

GR is GRE-binding competent but transactivation-incompetent in the GC-resistant cells of the mouse skin carcinogenesis model

Then, we tested whether GC-unresponsiveness of P6, B9, A5 and CarB cells is associated with reduced DNA binding of GR to GRE-containing targets. EMSAs were performed using nuclear cell extracts of C5N, P6, B9, A5 and CarB incubated with a ^{32}P-labelled double stranded oligonucleotide that contains a GRE binding site from the human metallothionin II promoter (hMTII-GRE). The binding of GR to GREs remains ligand-dependent only in the in the GC-sensitive C5N cell line (Fig. 3a), in contrast to the GC-resistant P6, B9, A5 and CarB cells, in which GR has acquired the ability to bind to GREs in the absence of dexamethasone (Fig. 3b). Additionally, the pattern of GR DNA binding along the five cell lines is consistent with their nuclear GR expression profile. This evidence indicates that the ability of GR to bind to GRE-containing targets through its DNA binding domain remains intact and proportional to the nuclear GR levels (Fig. 3b). Therefore, we plausibly hypothesized that although GR binds to target GREs in a ligand-independent manner in the P6, B9, A5 and CarB cells, it might be incapable of transactivating its targets, thus providing a reason for their GC-resistance. To

this end, we then tested whether unresponsiveness of GC-resistant cells to GCs might be associated with inability of GRE-bound GR to transactivate crucial antiproliferative targets. We used luciferase assays to monitor the ability of endogenous GR to activate the glucocorticoid-responsive enhancer of β-globin in the presence of dexamethasone in all mouse cell lines (Fig. 3c). Significant luciferase activity was observed only in the GC-sensitive C5N cells upon GC treatment. In parallel, using Q-PCR, we estimated the endogenous expression of the characteristic GC-responsive antiproliferative direct GR targets $p57^{KIP2}$ [31] and GILZ (Glucocorticoid-induced leucine zipper)[32] in dexamethasone-treated versus dexamethasone-untreated cells. In agreement with the luciferase assay findings, both targets were significantly induced ~~(t-test, $p<0.05$)~~ in the GC-responsive C5N cells upon dexamethasone treatment, whereas the corresponding levels were not upregulated after addition of dexamethasone in all GC-resistant cells (Fig. 3d and 3e). However, sequencing analysis revealed that this impairment is not attributed to direct mutations in the domains of the GR gene that are responsible for the GR transactivation function (Supplementary Material 1).

N-bromotaurine induces antiproliferative effects in GC-resistant cancer cell lines

Then, we checked whether BrTaur restores antiproliferative response in our model system. To this end, we treated cells with 25μM, 75μM, 125μM and 250μM BrTaur and subjected them to proliferation assays. This range is consistent with the therapeutic concentrations currently used, in the investigational clinical setting against inflammatory conditions and microbial infections.[13,15] BrTaur exerted a potent, dose-dependent antiproliferative effect in the GC-sensitive C5N and the GC-resistant P6, B9, A5 and CarB cells, which is evidenced from 125μM (Fig. 4a). The maternal substance taurine, from which BrTaur is produced upon reduction with HOBr, has been previously reported to exert anticancer properties[33,34]. Therefore, we treated cell lines with the concentration range of unbrominated taurine that corresponded to the tested concentration range of its brominated derivatives[23]. Taurine treatment did not affect mouse skin cancer cell proliferation in a significant, potent and consistent manner (Fig. 4b), implying that bromination of the taurine is the crucial factor for the consistent antiproliferative effect on cells. ANOVA utilising Dunetts' T3 post-hoc analysis demonstrated that P6 cells showed significant sensitivity at concentrations over 75μM bromotaurine. A5, B9 and CarB cell lines demonstrated sensitivity at concentrations over 125μM bromotaurine. The GC-responsive C5N cells are the least sensitive to bromotaurine. The BrTaur antiproliferative effect was reproduced in GC-resistant human cancer cells of epithelial origin, i.e. the prostate cancer cell line PC3 (Fig. 4c) and the breast cancer cell line MDA-MB-231 (Fig. 4d)[35,36] in the tested concentration range.

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N-bromotaurine inhibits cell cycle progressionin GC-resistant cells

Unlike dexamethasone (Fig. 5a), BrTaur induced antiproliferative effects on the GC-resistant, aggressive CarB cells in a concentration- and time- dependent manner (Fig. 5b). GCs inhibit cancer cell growth, at least in part, by blocking cell cycle at the G0/G1 phase. This ability of GCs to induce G1-arrest is often compromised in GC-resistant cancer cells.[30] In this context, we examined whether BrTaur bypasses lack of antiproliferative response in the GC-resistant cells by restoring G1-arrest. Using FACS analysis, we estimated the effect of three different BrTaur concentrations (125μM, 250μM and 500μM) on the G0/G1, S and G2/M phases, using the most aggressive GC-resistant cell line of our model system, i.e. CarB. Dexamethasone was used as the comparator substance and untreated cells were used as negative control. Dexamethasone was unable to induce G1-arrest, thus having an effect on cell cycle progression identical to the one observed for the GC-untreated cells. On the contrary, BrTaur in the concentrations of 125μM and 250μM enhanced the percentage of cells in G1 phase, thus simulating the effect of GCs on cell cycle. Interestingly, in the high, yet clinically physiological, concentration of 500μM, BrTaur potently affected both G1 and G2 phases, demonstrating a broader ability to target cell cycle. Its effect on the S phase is moderate and seems to be dose-dependent (Fig. 5c). The experiment was performed in triplicates and presented a p value <0.05 (t-test).

Ciplatin efficacy on GC-resistant cells is potentiated by N-bromotaurine: the earlier the initiation of N-bromotaurine co-administration, the more enhanced the synergistic effect

In clinical cancer therapeutics, GCs are routinely co-administered with cisplatin, either as adjuvant agents or to mitigate cisplatin adverse events. Therefore, for a substance to clinically qualify as a GC-substitute in the context of cancer, it should be able to enhance cisplatin's effects on tumor growth. To test if this applies for N-bromotaurine, we treated the GC-resistant aggressive spindle CarB cells of the mouse carcinogenesis model with a combination regimen of cisplatin plus N-bromotaurine. Three treatment schemes were used: a) pre-treatment, i.e. BrTaur 0-48h, followed by cisplatin 24-48h; b) concurrent treatment, i.e. BrTaur plus cisplatin, 0-48h; c) post-treatment, i.e. cisplatin 0-24h, followed by BrTaur 24-48h. Since the qualitative effect of BrTaur on cell cycle progression is dose-dependent for the higher 250μM (affects G1) and 500μM (affects both G1 and G2) concentrations (Fig. 5c), we tested both concentrations in the BrTaur-containing combination regimens. Each scheme was compared versus its corresponding comparator combination regimen of cisplatin plus 10^{-7}M dexamethasone. Strikingly, both BrTaur concentrations in all-three schemes synergized efficiently with 2.9μg/mL cisplatin (a value corresponding to the cisplatin concentration efficient to kill 27% of CarB cells; CarB IC50:3.7μg/mL) (Supplementary Material 2),

demonstrating significant superiority versus the corresponding comparator cisplatin plus dexamethasone regimens (Fig. 5d-f). ANOVA applying Dunnet's T3 post-hoc test showed that both BrTaur concentrations were efficient in all-three schemes ($p \leq 0.001$). The most potent synergistic effect was observed for the pre-treatment scheme, where both doses of 250μM and 500μM achieved similar efficacy. The synergistic effect was dose-dependent in concurrent treatment and post-treatment protocols. On the contrary, dexamethasone addition did not result to significant increase of the anti-proliferative effect of the regimen, either before, concurrently or following cisplatin treatment. Overall, the earlier the BrTaur co-administration started, the better its synergistic effect with cisplatin on CarB cell growth inhibition. Among the three treatment schemes, the inhibitory effect of cisplatin on cell growth was less potent in the post-treatment protocol; however higher doses of BrTaur were able to compensate for the delay of initiation of BrTaur co-administration (Fig 5f, fourth column).

DISCUSSION

Analogous to the microbes that develop a plethora of strategies to eventually become resistant to antibiotics, cancer cells invent several strategies to impair GR and overcome GC-sensitivity.[2] In several cases, a singleton cause of impairment cannot be identified, because GC-resistance is rather multifactorial and attributed to orchestrated inactivation of several GR-controlled pathways.[2,7,8] In this context, trying to identify impaired GR pathway(s) underlying GC-resistance and develop *de novo* a druggable molecule to restore responsiveness poses as a herculean task. A different approach to bypass GC-unresponsiveness of cancer cells would be to reposition alternatives from the pharmaceutical arsenal that are either approved or in late-stages of clinical trials for other GC-refractory inflammatory conditions. Given the emerging commonalities between inflammation and cancer, those alternatives might pose as latent substitutes of GCs' antiproliferative effect, awaiting in a "diamond-in-a-rough" state to be revisited in the context of cancer. This might decrease the pressure for natural selection of cancer cells that overcome the GC antiproliferative effects by deactivating their GR receptor and/or the GR-mediated pathways, the same way that prudent use of antibiotics or use of interchangeable antibiotics prevents the development of antibiotic-resistant microbe strains. Using GC-substitutes before ending-up prescribing GCs would also enable clinical oncologists to reserve the GC-based therapeutic options as a last-resort for aggressive tumors, without risking a possible induction of GC-resistance in earlier tumor stages.

To test this hypothesis we considered the mouse skin carcinogenesis system as our basal screening tool kit. Overall, the characteristics of our study system in terms of GC-sensitivity/GC-resistance and the underlying GR status are summarized in Table II. The model includes a GC-sensitive cell line C5N which retains a functional GR and can be used

as the positive, comparative screening control cell line of the panel. The rest of the cell lines represent GC-resistant papillomas, squamous and spindle cells. In terms of localization, we additionally observed a GR accumulation in the nucleus during transition from squamous-to-spindle stages, resulting to a mixed cytoplasmic and nuclear signal in spindle cells. This unexpected and previously unreported finding, which was reconfirmed in *in vivo* mouse spindle skin cancer tumors, indicates the possible existence of an heterogenous population of GR isoforms and/or variants, some of which may have dominant negative function to the typical full-length isoforms. From the pathology point of view, this means that GR nuclear localization may not be a positive clinical indication for GC-responsiveness, as originally had been suggested [37], especially given the fact that several dominant negative GR isoforms or splice variants that antagonize functional, full-length GR, and cause GC-unresponsiveness are also localized in the nucleus.[1,2]These issues will be clarified in future studies.

BrTaur, our first study case to be checked with our system, presents overlapping characteristics with GCs and is topically used in skin inflammatory conditions, such as acne vulgaris, instead of steroids [13]. It is well-tolerated and presents insignificant side-effects.[13,15] BrTaur surrogated for the antiproliferative effect on GC-sensitive and GC-resistant cells, thus providing the first evidence for its potential to be used interchangeably to GCs in the context of cancer, in the same concept they are currently clinically used interchangeably to GCs in the context of chronic inflammations and microbial infections. The fact that BrTaur efficiently synergizes with cisplatin to inhibit growth of GC-resistant cells further highlights its GC-mimicking therapeutic effect. The antiproliferative effect is strongly linked to the bromine moiety of the bromotaurine molecule and is mediated by inhibition of cell cycle in GC-resistant cells. The ability of BrTaur to produce a more consistent anticancer effect than taurine could be explained by the fact that the former is the oxidizing~~rapidly drastic~~ form, while the latter is the maternal, reservoir substance, considered as a pro-drug. In detail, taurine is retained in several tissues, primarily in liver, and is recruited in tissues undergoing oxidative stress by topically-produced HOCl and HOBr to finally be oxidized~~reduced~~ to its effective~~drastic~~ taurine haloamine derivatives. These scavenge the toxicity of the excess HOCl and HOBr and pick up the torch of immunologic responses at the lesion sites, preventing inflammation and exerting anti-microbial and oxidizing~~antioxidant like~~ properties[13]. In this respect, the inconsistent efficacy of taurine versus N-bromotaurine on the different cell lines of the mouse carcinogenesis system may be due to fluctuated micro-concentrations of HOBr in each different cell line milieu, thus resulting to corresponding fluctuations in the concentration of the active BrTaur finally being formed.

It should be noted that overproliferation in our system, as in actual tumors, is associated with deregulation of several main pathways in addition to GR transactivation impairment.[19] These pathways, such as the ERα and AP-1 oncogenic pathways, crosstalk

with GR since they are antagonized by its transrepression mode of action. Their progressive overactivation towards the aggressive stages in our system[22,26,38] implies a dysfunctional GR transrepression mode additionally to the demonstrated impairment of GR transactivation mode. Furthermore, the mixed cytoplasmic and nuclear signal detected in aggressive stages of skin cancer indicates the possible existence of a heterogeneous population of GR isoforms and/or variants, some of which may have dominant negative function to the typical full-length isoforms. This could be an additional reason for GR's inability to transactivate its targets and, thus, to subsequently mediate the antiproliferative effects of GCs in GC-resistant cells. This would mean that multiple factors causing GC-resistance are possibly accumulating towards the most aggressive stages. Therefore, our system must not be seen as a model dedicated to the study of a single GC-resistance cause. Rather, it should be cautiously used as a tool kit for performing preliminary screenings in order to discriminate the alternative agents with no antiproliferative action from the ones with the potential to restore antiproliferative response in GC-resistant cells.

Notably, although skin cancers are primarily associated with impairment of the GR/GC axis, they are not treated with GCs. Thus our model is not proposed as a means to spot GC-substitutes against this cancer type. However, it is the demonstrated ability of this skin cancer-based artificial model to produce results that are extrapolated to several other types of epithelial cancers[21], including the ones that are commonly treated with GCs, that gives this model an added value as an emerging generalized screening tool kit for identifying GC-substitutes. Using this basal screening tool, substances that are suspected, based on medical experience in the clinic, to have overlapping profiles with GCs could be confirmed as GC-substitutes before being repurposed for the management of cancer patients. Based on this screening tool, investigational N-bromotaurine was shown to act as a GC-substitute, while its effects where reproduced in cancer types that are commonly treated with GCs, such as the GC-resistant human breast and prostate cancer cell lines. Further confirmation of this anti-tumor effect in experimental animals in future studies could accelerate subsequent repositioning of BrTaur for the management of clinical cancer patients.

The point our approach highlights is that already-in-clinical-use compounds, able to consistently induce an antiproliferative phenotype in GC-resistant cells, even via molecular circuits which are currently as unknown to molecular biologists, as are unfamiliar to the GC-resistant cancer cells themselves, warrant to be unveiled and considered as possible GC-substitute therapeutic solutions. Analogous with what happens with newer generation antibiotics that catch the microbes unawares, the success of such compounds to startle cancer cells towards an antiproliferative direction may rely to their possible ability to act through pathways that the cancer cells have never been called to deactivate before in order to become aggressive. Such GC-substitutes may have the clinical potential to prolong

disease free-survival, reduce tumor size, delay progression, mitigate side-effects and improve quality of life, in combination with well-established drugs, thus complying to the FDA's established guidelines on cancer clinical trial end-points. (http://www.fda.gov/downloads/Drugs/Guidances/ucm071590.pdf).

Last but not least, burning issues regarding BrTaur mechanism of action are awaiting to be thoroughly addressed in future studies. Potential ways for its systemic administration in the context of cancer therapeutics should also be explored. Anti-inflammatory effects of BrTaur are mediated by modification of the IkappaBalpha, an NF-kappaB inhibitor[39]. This interaction could also underlie BrTaur antiproliferative effects on GC-resistant cells, given that NF-kappaB stands in the cross-roads between inflammation and cancer[40] and is a crucial effector of GR-mediated tumor-suppressor effects[2]. Given the commonalities shared between anti-inflammatory and anti-cancer pathways, BrTaur may, at least in part, exert its anti-cancer action, in addition to its well-known anti-inflammatory properties[13] by targeting common networks underlying both pathological entities[17,18]. Comprehensive, high-throughput analyses of the molecular and cellular changes and the transcriptional programs alterations triggered upon BrTaur treatment using state-of-the-art, multiomics approaches are anticipated to shed more light on this issue in the future. The full range of BrTaur functions might extend beyond interfering with GR-mediated pathways, and is currently under investigation. The role of BrTaur in cancer emerges as a subject of fruitful research and poses as a mysterious "black box", the decoding of which might pave the way for next generation therapeutics.

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FIGURE LEGENDS

Figure 1: Estimation of responsiveness of cell lines of the mouse skin carcinogenesis model to GCs (dexamethasone) by crystal violet assays. **(a)** In the representative time point of 48h, the ~~percentage~~ number of cells drastically decreases proportional to dexamethasone concentration in comparison with the untreated cells in the C5N cell line (expressed as ratio to untreated cells). In contrast, there is no change in cell numbers of dexamethasone-treated P6, B9, A5 and CarB cells compared to their corresponding untreated controls, in any of the concentrations tested. **(b)** The profile of the antiproliferative action of GCs on the GC-sensitive C5N cells over time, for each of the tested dexamethasone concentrations in comparison to a selected GC-resistant cell line P6. The experiments were performed in triplicates.

Figure 2: GR is translocated in nucleus during transition from squamous to spindle stage of skin carcinogenesis. **(a)** GR protein expression levels of total (tGR), cytoplasmic (cGR) and nuclear (nGR) extracts of the mouse skin carcinogenesis model. **(b)** ~~Quantification of~~ Western blot results indicate~~s~~ a switch in the cytoplasmic-to-nuclear GR ratio (cGR-to-nGR) during transition from squamous to spindle stage. **(c)** Immunocytochemistry analysis of the panel of mouse skin carcinogenesis model with anti-GR antibody revealed cytoplasmic staining in C5N, P6 and B9 cells, and mixed cytoplasmic and nuclear staining in A5 and CarB cells. The black arrows indicate GR-stained nuclei. These immunocytochemical patterns were rather uniform in the above mentioned cell lines. **(d)** The immunofluorescence GR signal is cytoplasmic in the squamous B9 cells, but nuclear in the spindle A5 cells. **(e)** Immunohistochemistry using anti-GR antibody in paraffin-embedded tissues isolated from the papilloma, squamous and spindle stage of the same chemically-induced tumor in one mouse revealed higher number of GR-stained nuclei in spindle cells in comparison with the corresponding papilloma and squamous stage. Each figure represents a 200x magnitude, whereas the upper-right boxes in each figure represent a 400x magnitude. The black boxes represent the magnified area. The black arrows indicate GR-stained nuclei.

Figure 3: GR is DNA binding-competent but transactivation-incompetent in GC-resistant cell lines of the mouse skin carcinogenesis model. **(a)** Electrophoretic mobility shift assay in the GC-responsive C5N revealed binding activity of GR to glucocorticoid responsive elements (GRE) only upon dexamethasone (C5N+dex) treatment. **(b)** EMSA assay for GR using nuclear extracts demonstrated binding activity of GR to glucocorticoid responsive elements (GRE) of the GC-unresponsive P6, B9, A5 and CarB cells in the absence of dexamethasone signal. DNA binding increases directly proportional to the demonstrated nuclear GR protein

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levels towards more aggressive cell lines. Specificity of the EMSA reaction is confirmed by supershift reactions in A5 (lane 6) cells using anti-GR antibody. **(c)** Luciferase assays in the GC-sensitive and the GC-resistant cells of the mouse skin carcinogenesis model co-transfected with the 17m-GRE-G-Luc, in dexamethasone absence (-dex) or presence (+dex). Luciferase expression is significantly induced (One asterisk (*) denotes p <0.05) only in the GC-sensitive C5N cells upon dexamethasone treatment. Error bars represent the standard error of the mean. Two-tailed p values are are derived from independent t-tests. Three biological replicates were available for each experiment. **(d)** Box plots demonstrating the transcription induction of GILZ under dexamethasone treatment in ~~Q-PCR estimation of GILZ levels~~ in the GC-sensitive and the GC-resistant cell lines before (-dex) and after (+dex) dexamethasone treatment, estimated by qPCR. The relative quantification (RQ) values in $\log_{10}$ are shown. For each cell line, the untreated control was used as calibrator. The housekeeping gene beta-2 microglobulin was used as an internal control of mRNA Q-PCR expression. The experiments were performed in triplicates. **(e)** Same as 3d, for $p57^{KIP2}$ mRNA levels. ~~.~~In both genes, significant induction is only observed in C5N cells (indicated by star). RQ: relative quantification value.

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Figure 4: Estimation of the antiproliferative response of the GC-sensitive and the GC-resistant cell lines to N-bromotaurine by proliferation assays. **(a)** In the representative time point of 48h, the ~~percentage~~ number of cells drastically decreases in comparison with the corresponding untreated control cells for all cell lines of the study model (expressed as ratio to untreated cells). **(b)** Comparative treatment of the cells with the maternal substance taurine did not affect mouse skin cancer cell proliferation in a significant and consistent manner, implying that bromination of the taurine is the crucial factor for the consistent antiproliferative effect on cells. **(c)** The profile of the antiproliferative action of N-bromotaurine on the GC-resistant GC-resistant prostate cancer PC3. **(d)** Same as 4c~~.~~ for the human GC-resistant prostate cancer MDA-MB-231. The experiments were performed in triplicates.

Figure 5: Comparison of the antiproliferative response of the aggressive GC-resistant cells to GCs versus N-bromotaurine. **(a)** proliferation assays on CarB cells treated with dexamethasone reveal lack of significant antiproliferative response of CarB upon addition of GCs over time, as evidenced by the OD measurements. **(b)** In contrast, proliferation assays on CarB cells treated with N-bromotaurine instead of dexamethasone reveal restoration of antiproliferative response of CarB cells by N-bromotaurine over time. The restoration of antiproliferative response is statistically significant~~potent~~ in the 250μM concentration, after 48 and 72 hours of N-bromotaurine treatment. Three independent experiments were performed,

each in triplicate. ~~Mean values were significantly different from those of the untreated controls in the N-bromotaurine treated cells, but not in the dexamethasone treated cells.~~ The means+SEM values are shown in the graphs.~~.~~ **(c)** FACS analysis of N-bromotaurine-treated CarB cells (CarB + BrTaur) compared with dexamethasone-treated CarB cells (CarB + dex) and untreated CarB cells (CarB). BrTaur, at concentrations of 125μM and 250μM, ~~-~~increased the number of cells in G0/G1 phase; whereas at the high concentration of 500μM, BrTaur drastically decreased both G0/G1 and G2/M phases. Dexamethasone, at 10^{-7}M concentration failed to significantly affect the number of cells in G1/G0 phase. Corresponding summary graphs for each phase of the cell cycle (means±SEM from 3 independent FACS analyses measurements) derived by comparison of untreated CarB cells each of the dexamethasone-treated, 125μM BrTaur-treated, 250μM BrTaur-treated and 500μM BrTaur-treated CarB cells~~,~~ ~~using t test~~. **(d-f)** 250μM BrTaur-treated and 500μM BrTaur-treated CarB cells synergized efficiently with 2.9 cisplatin (cis) after 48hours of BrTaur plus cisplatin combination therapy as **(d)** pre-treament (BrTaur + cis), **(e)** concurrent treatment (cis/BrTaur) or **(f)** post-treatment (cis + BrTaur)~~, t test, p value <0.05~~. Results are flagged with no asterisk when p-value is more than 0.05, and with two~~an~~ asterisk~~s~~ when the p-value is less than 0.001~~5~~.~~ ~~(See text for details on treatment schemes of 5d-f).

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N-bromotaurine surrogates for loss of antiproliferative response and enhances cisplatin efficacy in cancer cells with impaired glucocorticoid receptor

Stella Logotheti[1,7], Nikolas Khoury[1], Spiros A. Vlahopoulos[2], Elena Skourti[1], Dimitra Papaevangeliou[1], Triantafyllos Liloglou[3], Vassilis Gorgoulis[4], Irina Budunova[5], Anthony M. Kyriakopoulos[6], Vassilis Zoumpourlis[1*]

[1]Biomedical Applications Unit, Institute of Biology, Medicinal Chemistry and Biotechnology, National Hellenic Research Foundation, 48 Vassileos Constantinou Avenue, 116 35 Athens, Greece.
[2]Horemio Research Institute, First Department of Pediatrics, "Aghia Sophia" Children's Hospital, National and Kapodistrian University of Athens, Athens, Greece.
[3]University of Liverpool, Department of Molecular and Clinical Cancer Medicine, 200 London Road, Liverpool L3 9TA, UK
[4]Laboratory of Histology-Embryology, Molecular Carcinogenesis Group, Medical School, National and Kapodistrian University of Athens, Athens, Greece.
[5]Department of Dermatology, Northwestern University, Chicago, IL, USA.
[6]Nasco AD, Biotechnology Laboratory, 11th Sachtouri str., Piraeus, Greece.
[7]Department of Biotechnology, Agricultural University of Athens, Athens, Greece

* Corresponding author
Tel: 30-10-7273730
Fax: 30-10-7273677
e-mail: vzub@eie.gr

Running title : Repurposing BrTaur in GC-resistant cancers

Keywords: GR; glucocorticoid resistance; N-bromotaurine; skin carcinogenesis; drug re-purposing

Abbreviations: GC: glucocorticoid; GR: glucocorticoid receptor; GRE: glucocorticoid receptor element; NSAID: non-steroid anti-inflammatory drug; BrTaur: N-bromotaurine; ClTaur: N-chlorotaurine; EMSA: electrophoretic mobility shift assay; FACS: fluorescence-activated cell sorting; qRT-PCR: quantitative Real-Time-Polymerase Chain Reaction.

ABSTRACT

Glucocorticoids (GCs) are frequently used in anticancer combination regimens; however, their continuous use adds selective pressure on cancer cells to develop GC-resistance via impairment of the glucocorticoid receptor (GR), therefore creating a need for GC-alternatives. Based on the drug repurposing approach and the commonalities between inflammation and neoplasia, drugs that are either in late-stage clinical trials and/or already marketed for GC-refractory inflammatory diseases, could be evaluated as GC-substitutes in the context of cancer. Advantageously, unlike new molecular entities currently being *de novo* developed to restore GC-responsiveness of cancer cells, such drugs have documented safety and efficacy profile, which overall simplifies their introduction in clinical cancer trials. In this study, we estimated the potential of a well-established, multistage, cell line-based, mouse skin carcinogenesis model to be exploited as an initial screening tool for unveiling covert GC-substitutes. First, we categorized the cell lines of this model to GC-sensitive and GC-resistant, in correlation with their corresponding GR status, localization and functionality. We found that GC-resistance starts in papilloma stages, due to a dysfunctional GR, which is overexpressed, DNA binding-competent, but transactivation-incompetent in papilloma, squamous and spindle stages of the model. Then, aided by this tool, we evaluated the ability of N-bromotaurine, a naturally-occurring, small-molecule, NSAID which is under consideration for use interchangeably/in replacement to GCs in skin inflammations, to restore antiproliferative response of GC-resistant cancer cells. Unlike GCs, N-bromotaurine inhibited cell-cycle progression in GC-resistant cancer cells and efficiently synergized with cisplatin, thus indicating a potential to be exploited instead of GCs against cancer.

INTRODUCTION

Glucocorticoids (GCs) are steroid hormones which inhibit tumor cell proliferation, mitigate chemotherapy side effects and enhance efficiency of anticancer agents[1,2]. They are frequently included in combination anticancer therapies, either as palliative agents against chemotherapy-induced-nausea-and-vomiting or as antiproliferative agents. Their effects are mediated by the glucocorticoid receptor (GR). GC binds to GR which, following dissociation from a cytoplasmic chaperone/co-chaperone complex, translocates to the nucleus, homodimerizes and regulates gene expression. GR transactivates or transrepresses genes by direct binding to GREs (glucocorticoid responsive elements), by tethering itself to other transcription factors apart from DNA binding, or in a composite manner by both direct GRE binding and interactions with transcription factors bound to neighboring sites (reviewed by Ramamoorthy et al[2]). Thus, a functional GR elicits tumor-suppressive events in a pleiotropic manner, through a plethora of mechanisms and crucial pathways.[3-5]

Theoretically, the clinical benefits demonstrated by the long-term experience on GCs, combined with the tumor-suppressive nature of GR, offer a strong alibi for their routine use in cancer therapeutics (e.g. prostate cancer, breast cancer, leukemia), as evidenced by the increasing number of GC-containing combination regimens, both established and investigational. Nevertheless, GC cotreatment often induces resistance towards cancer therapy[1,6] thus raising concerns regarding the tendency of GC-sensitive cancer cells to develop resistance upon frequent GC use. Mechanistically, this tendency is attributed to the pleiotropic nature of GR *per se*. GR's pleiotropy becomes a double-edged sword, since cancer cells have, at their disposal, as many potentials to overcome GR's antiproliferative barrier and achieve GC-resistance, as is the plethora of underlying GR-mediated antiproliferative pathways they can defuse one way or another. For neutralizing GR antiproliferative effects, cancer cells exploit several strategies, which in several cases simultaneously co-exist in a cancer cell (e.g. reduced GR expression, reduced DNA binding ability, GR mutations and polymorphisms, co-expression of dominant negative GR isoforms, impairment of transrepression mode et.c.).[2,7,8] GC-unresponsiveness by GR impairment may start as early as the benign stages, highlighting that inactivation of GC/GR axis is a selective advantage in order for a cancer cell to surpass the antiproliferative break and continue its tumorigenic march towards aggressive stages.[9]

This overall necessitates alternatives restoring and/or surrogating for GCs' antiproliferative effects.[2,10] To this end, innovative synthetic molecules are being developed by academia, aiming to re-activate GR-mediated antiproliferative pathways and restore GC-responsiveness of cancer cells[10]. If they successfully pass clinical trials, they will eventually find their way to the bedside. But this established bench-to-bedside pipeline is not an one-way street. The recently framed concept of drug repurposing suggests evaluating suitability of

known drugs for use in new indications[11]. Implementing this concept in the issue of GC-resistance, we postulate that drugs which are already in late stages of clinical trials or approved for indications other than cancer may be latent GC-substitutes, able to imitate aspects of the GC therapeutic profile. These hypothetical latent GC-substitutes might be competent to either restore the antiproliferative phenotypes in GC-resistant cells; or to be used interchangeably to GCs, in order to reduce the selective pressure exerted by continuous use of GCs on GC-sensitive cells, thus preventing/delaying their clonal expansion to GR-impaired and, hence, GC-resistant cells. A clinical advantage of the proposed approach is that these candidate substances are actually closer to the bedside than they are to the bench, since they have a more characterized efficacy and safety profile, in terms of their documentation for other therapeutic indications. This is translated to both faster filing and regulatory approval procedures and to reduced financial costs to develop these substances as anticancer agents, compared to starting the anticancer drug research-and-development workflow from scratch, i.e. by developing New Molecular Entities (NMAs, defined by FDA as experimental substances without precedent among regulated and approved drug products).[11]

Except for anticancer agents, GCs are also common anti-inflammatory agents. In fact, their use against inflammations historically preceded their use against neoplasias. GC-resistance is a frequent problem in inflammations as well, and drugs are being developed in replacement of GC-containing anti-inflammatory regimens.[12] Emerging GC-substitutes that are currently in the investigational clinical setting for inflammation management include, but are not limited to, the NSAID (Non-Steroid Anti-Inflammatory Drug) taurine haloamine derivatives, mainly N-bromotaurine (BrTaur) and N-chlorotaurine (ClTaur). These are generated by eosinophils and neutrophils at a site of inflammation and exert potent anti-inflammatory properties. Other common features shared with GCs is their naturally-occurring and small-molecule nature and their immunomodulatory and antimicrobial properties[13]. Another common characteristic is the potential to ameliorate chemotherapy-induced nausea and vomiting, since their maternal substance, i.e. the non-essential aminoacid taurine, which is orally administered as a pro-drug in order to be converted to the haloamine derivative at the site of inflammation, was recently proven clinically capable of such an effect in leukemic patients.[14] Advantageously, taurine haloamines have shown good efficacy, tolerance and insignificant toxic effects upon topical use on clinical patients who are refractory to conventional GC-based anti-inflammatory therapies[13,1516]. Based on the association between chronic inflammatory diseases and neoplasias,[17,18] we postulated that a drug which exerts overlapping features and common therapeutic indications with GCs and is able to surrogate for GCs in GC-resistant inflammations may be able to surrogate for the GCs' antiproliferative function in GC-resistant cancer cells as well.

As a springboard for testing this hypothesis, we took advantage of our long-term experience on a well-established mouse model of skin carcinogenesis.[19] This comprises of a series of cell lines which represent different stages of mouse skin tumor progression and are categorized on the basis of increasing aggressiveness to immortalized keratinocytes (C5N), benign papillomas (P6), malignant squamous carcinomas (B9), and highly invasive spindle cells (A5, CarB).[19] The B9:A5 pair represents the clonal expansion from squamous to spindle stages. The model has been developed in Dr. A. Balmain's lab and has been thoroughly reviewed[18] and described previously.[20] Briefly, in order to obtain these cell lines, each of which represent the initiation, promotion or progression stages of skin carcinogenesis, a chemical carcinogenesis protocol on mice was applied. The normal epidermis of mice or normal epithelial mouse cells were treated with a single dose of the polycyclic aromatic hydrocarbon 7,12 dimethylbenz[a]anthracene (DMBA), followed by weekly applications of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). This led to the development of numerous benign papillomas, some of which progressed to malignant squamous cell carcinomas several weeks after the first exposure to carcinogens and cell lines were produced from these tumors. Overall, this model poses the following advantages: a) it is multistage, meaning that it simulates the step-wise manner by which a tumor initiates, promotes and progresses, b) it is coherent, since cell lines have been derived in a consistent manner following a meticulous chemical carcinogenesis protocol, c) although skin-tissue based, it further applies to almost all epithelial cancers.[21] Furthermore, the fact that the model is skin tissue-based facilitates our analysis, because skin cancer is a traditional field where the mechanisms of GR function in correlation to GC-responsiveness have been adequately studied.[4,5,9] Therefore, although our model has never been characterized before in terms of GC-responsiveness, it stood a good chance to faithfully mirror or even complement previous robust findings.

First, we characterized our system in terms of antiproliferative response to GCs. Then, we correlated this responsiveness to the underlying GR expression status, localization and functionality. Finally, we checked the ability of BrTaur to bypass GC-resistance of cancer cells either alone or in combination with cisplatin.

MATERIALS AND METHODS

Cells and culture conditions

Mouse cell lines of the mouse skin carcinogenesis model have been produced and obtained by Dr. Allan Balmain. All human cell lines used were obtained by American Type Culture Collection (ATCC). Cells were cultured as previously described.[22] The preparation[23] and use

of BrTaur is covered by licensing agreement. The in-house formulation was donated by NASCO AD Biotechnology Laboratory for preclinical research purposes.

Proliferation assays

Collectively, 2250 cells per well were seeded in 96-well plates. After cells were attached, the first measurement was taken. This time point is called 0 hours. At this point, 24h after seeding, dexamethasone (10^{-9} – 10^{-6}M, from a stock of 10^{-3}M dexamethasone diluted in ethanol), BrTaur (25μM-250μM from a stock of 4mM BrTaur) or taurine (5-50mM from a stock of 200mM taurine diluted in water) was added and measurements were taken after 24, 48 and 72h. For co-treatment experiments, cells were treated with either 10^{-7}M dexamethasone or BrTaur (125, 250 or 500μM) 24-hours prior, con-currently with or 24-hours post cisplatin treatment. Untreated cells were used as controls. Following treatments, cells were fixed with 100% methanol and, then, crystal violet solution was added to each well. After a 10-minute incubation in room temperature, each well was washed 3 times with 200μL water, and plates were incubated on a shaker for 45min. Optical Density (OD) was measured at 595nm using a Tecan reader. The data was transferred to Microsoft Excel and analyzed. Background absorbance was corrected using triplicate sets of wells containing medium only (no cells) and crystal violet reagent as per experimental well. Three independent experiments were performed and each one of them included a triplicate value set.

RNA extraction, cDNA synthesis and qRT-PCR analysis

mRNA extraction, cDNA synthesis and quantitative real-time PCR was conducted as previously described.[24] Primers used appear in Table I.

Preparation of cell lysates and Western blot analysis

Total, cytoplasmic and nuclear cell lysates were prepared as previously described.[25] The primary antibody was an in-house anti-GR rabbit polyclonal antibody, clone 2F8, against aminoacids 305-427 of the N-terminal domain (kindly provided by Dr. M.N. Alexis), in a 1:500 dilution. Primary anti-beta-actin antibody in a 1:1000 dilution was used as a loading control. The secondary antibody was a mouse anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in a 1:1000 dilution.

Two-stage chemical carcinogenesis protocol

Tumors induced on mouse skin following a chemical carcinogenesis protocol were fixed and paraffin-embedded. Slides carrying formalin-fixed paraffin-embedded (FFPE) mouse skin papilloma and squamous and spindle tumors were prepared as previously described.[22] In

vivo experiments were performed in the in-house authorized animal house. Experiments complied with the Protocol on the Protection and Welfare of Animals, as obliged by the rules of the National Hellenic Research Foundation, the regulations of the National Bioethics Committee and the article 3 of the presidential decree 160/1991 (in line with 86/609/EEC directive) regarding the welfare of experimental animals.

Immunohistochemical and immunocytochemical staining

Immunohistochemical staining on FFPE sections was performed as described earlier.[26] The sections were stained with anti-GR, clone 2F8, in a 1:10 dilution. For the immunocytochemistry staining with anti-GR antibody 2F8, we followed the same procedure, incubation periods and reagents, by omitting the deparaffinization step.

Immunofluorescence staining

Cells were grown and fixed on coverslips and where subjected to immunofluorescence staining as previously described.[27] The slides were incubated with primary antibody anti-GR, clone M-20 (Santa Cruz Biotechnology, Santa Cruz, CA) in a 1:50 dilution. The secondary antibody was anti-rabbit FITC-conjugated (Jackson Laboratory, Bar Harbor, Maine, USA) diluted in 1:100.

Electrophoretic Mobility Shift assay

Annealed oligonucleotides for the *human metallothionin IIA* Glucocorticoid Responsive Element (5'-TGGTACACTGTGTCCTGAATTCA-3' and 5'-TGAATTCAGGACACAGTGTACCA-3') were end-labeled with γ^{32}P-ATP using T4-polynucleotide kinase and the reaction products were purified on a 8% polyacrylamide gel. DNA binding reactions were performed as previously described.[28] For the supershift control experiment, the primary polyclonal antibody anti-GR 10-10 (kindly provided by Dr. M. Alexis) was used.

Plasmids, transfections and luciferase reporter assay

A luciferase plasmid carrying GRE sequences (17m-GRE-G-Luc), as well as a control vector carrying no GRE binding site (tata-pG13Luc) described previously[29] were used for transfections. Where indicated, cells were incubated with dexamethasone and transfected with by the calcium phosphate method, as described previously[7]. The luciferase activity was measured using a luminometer and was normalized for transfection efficiency with the β-galactosidase activity.

FACS analysis

Cells were harvested, trypsinized and centrifuged at 1,000rpm for 5min, at room temperature. The pellet was resuspended in 500μL PBS, fixed with 80% ethanol, vortexed, and stained with propidium iodide (50μg/mL), in the presence of 5mmol/L $MgCl_2$ and 10μg/mL RNase A in 10mmol/L Tris-HCl (pH 7.5). DNA content was analyzed on a FACSCalibur (Becton Dickinson) using the Modfit software.

Statistical analysis

Data are expressed as mean±SD. Each experiment was performed in triplicates. Then, the triplicate set values of three independent experiments were analyzed. For statistical analyses of proliferation assays results, ANOVA utilising Dunetts' T3 post-hoc analysis was applied. QPCR results were evaluated using Mann-Whitney's test. Luciferase assays were analyzed using independent student's t-tests. P values of less than 0.05 were considered significant.

RESULTS

The antiproliferative effect of GCs is lost in the promotion and progression stages of mouse skin carcinogenesis

Our first priority was to explore the GC antiproliferative effect on the cell lines of our system. GC effect ranges from proliferative in very low concentrations, to cytostatic/antiproliferative in more physiological concentrations and cytotoxic/apoptotic in higher concentrations.[30] To monitor GC effect on our system, we treated cells with a range of dexamethasone concentrations previously demonstrated to show antiproliferative effects on mouse keratinocytes (10^{-9}M - 10^{-6}M),[9] and subjected them to proliferation assay. Consistent with previous similar findings,[9] only the immortalized C5N cells were growth-inhibited by dexamethasone, in a concentration-dependent manner. P6, B9, A5 and CarB cells continued to proliferate despite dexamethasone presence (Fig. 1a). Dexamethasone induced no effect in P6, B9, A5 and CarB cells, neither proliferative nor antiproliferative. On the other hand, each tested dexamethasone concentration reduced proliferation rate of the GC-responsive C5N cells in a time-dependent manner (Fig. 1b). ANOVA utilising Dunetts' T3 post-hoc analysis demonstrated that C5N cells showed significant sensitivity to dexamethasone. In particular, at 72 hours, significant loss of survival was observed at 10^{-8}, 10^{-7}, and 10^{-6} concentrations ($p=0.014$, $p=0.002$, $p<0.001$ respectively). On the contrary, no significant difference observed between the untreated and treated P6 cells at any dexamethasone concentration (Fig. 1b). Therefore, the cells of our system were categorized to GC-sensitive (C5N; susceptible to growth inhibition by GCs) and to GC-resistant (P6, B9, A5 and CarB; no response to GCs, neither proliferative nor antiproliferative).

GR expression and localization status in the multistage mouse skin carcinogenesis model

Then, we monitored GR expression and localization in each cell line of our model. First, western blot revealed an elevation of total GR levels towards more aggressive cancer stages. Nuclear GR levels gradually increase, showing an abrupt increase during B9-to-A5 transition, whereas there is a reduction of cytoplasmic GR protein levels from B9 to A5 cells, documenting a switch of the cytoplasmic-to-nuclear ratio upon B9/A5 transition, which is independent from GC presence (Fig. 2b). This tendency of GR to translocate to the nucleus during squamous-to-spindle transition was further confirmed by immunocytochemistry, which revealed cytoplasmic GR localization in C5N, P6 and B9 cells, and mixed cytoplasmic-nuclear localization in A5 and CarB cells (Fig. 2c). The GR nucleocytoplasmic translocation at the squamous-to-spindle threshold was additionally confirmed by immunofluorescence in B9 and A5 cells, which revealed a clear cytoplasmic signal in B9 cells, but an intense nuclear staining in A5 cells (Fig. 2d). To the best of our knowledge, this mixed cytoplasmic and nuclear GR localization in aggressive stages has never been reported before. To exclude the possibility that this observation is a cell-line artefact, we confirmed it immunohistochemically *in vivo*, on sections from skin tumors induced in mice following a chemical carcinogenesis protocol. Indeed, on tumors of the same animal, papilloma stage presents mainly cytoplasmic GR localization whereas in the corresponding squamous stage GR localization is more intense and gets even more intense in the spindle stage-tumors (Fig. 2e).

GR is GRE-binding competent but transactivation-incompetent in the GC-resistant cells of the mouse skin carcinogenesis model

Then, we tested whether GC-unresponsiveness of P6, B9, A5 and CarB cells is associated with reduced DNA binding of GR to GRE-containing targets. EMSAs were performed using nuclear cell extracts of C5N, P6, B9, A5 and CarB incubated with a ^{32}P-labelled double stranded oligonucleotide that contains a GRE binding site from the human metallothionin II promoter (hMTII-GRE). The binding of GR to GREs remains ligand-dependent only in the in the GC-sensitive C5N cell line (Fig. 3a), in contrast to the GC-resistant P6, B9, A5 and CarB cells, in which GR has acquired the ability to bind to GREs in the absence of dexamethasone (Fig. 3b). Additionally, the pattern of GR DNA binding along the five cell lines is consistent with their nuclear GR expression profile. This evidence indicates that the ability of GR to bind to GRE-containing targets through its DNA binding domain remains intact and proportional to the nuclear GR levels (Fig. 3b). Therefore, we plausibly hypothesized that although GR binds to target GREs in a ligand-independent manner in the P6, B9, A5 and CarB cells, it might be incapable of transactivating its targets, thus providing a reason for their GC-resistance. To this end, we then tested whether unresponsiveness of GC-resistant cells to GCs might be

associated with inability of GRE-bound GR to transactivate crucial antiproliferative targets. We used luciferase assays to monitor the ability of endogenous GR to activate the glucocorticoid-responsive enhancer of β-globin in the presence of dexamethasone in all mouse cell lines (Fig. 3c). Significant luciferase activity was observed only in the GC-sensitive C5N cells upon GC treatment. In parallel, using Q-PCR, we estimated the endogenous expression of the characteristic GC-responsive antiproliferative direct GR targets $p57^{KIP2}$ [31] and GILZ (Glucocorticoid-induced leucine zipper)[32] in dexamethasone-treated versus dexamethasone-untreated cells. In agreement with the luciferase assay findings, both targets were significantly induced in the GC-responsive C5N cells upon dexamethasone treatment, whereas the corresponding levels were not upregulated after addition of dexamethasone in all GC-resistant cells (Fig. 3d and 3e). However, sequencing analysis revealed that this impairment is not attributed to direct mutations in the domains of the GR gene that are responsible for the GR transactivation function (Supplementary Material 1).

N-bromotaurine induces antiproliferative effects in GC-resistant cancer cell lines

Then, we checked whether BrTaur restores antiproliferative response in our model system. To this end, we treated cells with 25μM, 75μM, 125μM and 250μM BrTaur and subjected them to proliferation assays. This range is consistent with the therapeutic concentrations currently used, in the investigational clinical setting against inflammatory conditions and microbial infections.[13,15] BrTaur exerted a potent, dose-dependent antiproliferative effect in the GC-sensitive C5N and the GC-resistant P6, B9, A5 and CarB cells, which is evidenced from 125μM (Fig. 4a). The maternal substance taurine, from which BrTaur is produced upon reduction with HOBr, has been previously reported to exert anticancer properties[33,34]. Therefore, we treated cell lines with the concentration range of unbrominated taurine that corresponded to the tested concentration range of its brominated derivatives[23]. Taurine treatment did not affect mouse skin cancer cell proliferation in a significant, potent and consistent manner (Fig. 4b), implying that bromination of the taurine is the crucial factor for the consistent antiproliferative effect on cells. ANOVA utilising Dunetts' T3 post-hoc analysis demonstrated that P6 cells showed significant sensitivity at concentrations over 75μM bromotaurine. A5, B9 and CarB cell lines demonstrated sensitivity at concentrations over 125μM bromotaurine. The GC-responsive C5N cells are the least sensitive to bromotaurine,. The BrTaur antiproliferative effect was reproduced in GC-resistant human cancer cells of epithelial origin, i.e. the prostate cancer cell line PC3 (Fig. 4c) and the breast cancer cell line MDA-MB-231 (Fig. 4d)[35,36] in the tested concentration range.

N-bromotaurine inhibits cell cycle progressionin GC-resistant cells

Unlike dexamethasone (Fig. 5a), BrTaur induced antiproliferative effects on the GC-resistant, aggressive CarB cells in a concentration- and time- dependent manner (Fig. 5b). GCs inhibit cancer cell growth, at least in part, by blocking cell cycle at the G0/G1 phase. This ability of GCs to induce G1-arrest is often compromised in GC-resistant cancer cells.[30] In this context, we examined whether BrTaur bypasses lack of antiproliferative response in the GC-resistant cells by restoring G1-arrest. Using FACS analysis, we estimated the effect of three different BrTaur concentrations (125μM, 250μM and 500μM) on the G0/G1, S and G2/M phases, using the most aggressive GC-resistant cell line of our model system, i.e. CarB. Dexamethasone was used as the comparator substance and untreated cells were used as negative control. Dexamethasone was unable to induce G1-arrest, thus having an effect on cell cycle progression identical to the one observed for the GC-untreated cells. On the contrary, BrTaur in the concentrations of 125μM and 250μM enhanced the percentage of cells in G1 phase, thus simulating the effect of GCs on cell cycle. Interestingly, in the high, yet clinically physiological, concentration of 500μM, BrTaur potently affected both G1 and G2 phases, demonstrating a broader ability to target cell cycle. Its effect on the S phase is moderate and seems to be dose-dependent (Fig. 5c). The experiment was performed in triplicates and presented a p value <0.05 (t-test).

Ciplatin efficacy on GC-resistant cells is potentiated by N-bromotaurine: the earlier the initiation of N-bromotaurine co-administration, the more enhanced the synergistic effect

In clinical cancer therapeutics, GCs are routinely co-administered with cisplatin, either as adjuvant agents or to mitigate cisplatin adverse events. Therefore, for a substance to clinically qualify as a GC-substitute in the context of cancer, it should be able to enhance cisplatin's effects on tumor growth. To test if this applies for N-bromotaurine, we treated the GC-resistant aggressive spindle CarB cells of the mouse carcinogenesis model with a combination regimen of cisplatin plus N-bromotaurine. Three treatment schemes were used: a) pre-treatment, i.e. BrTaur 0-48h, followed by cisplatin 24-48h; b) concurrent treatment, i.e. BrTaur plus cisplatin, 0-48h; c) post-treatment, i.e. cisplatin 0-24h, followed by BrTaur 24-48h. Since the qualitative effect of BrTaur on cell cycle progression is dose-dependent for the higher 250μM (affects G1) and 500μM (affects both G1 and G2) concentrations (Fig. 5c), we tested both concentrations in the BrTaur-containing combination regimens. Each scheme was compared versus its corresponding comparator combination regimen of cisplatin plus 10^{-7}M dexamethasone. Strikingly, both BrTaur concentrations in all-three schemes synergized efficiently with 2.9μg/mL cisplatin (a value corresponding to the cisplatin concentration efficient to kill 27% of CarB cells; CarB IC50:3.7μg/mL) (Supplementary Material 2), demonstrating significant superiority versus the corresponding comparator cisplatin plus

dexamethasone regimens (Fig. 5d-f). ANOVA applying Dunnet's T3 post-hoc test showed that both BrTaur concentrations were efficient in all-three schemes ($p \leq 0.001$). The most potent synergistic effect was observed for the pre-treatment scheme, where both doses of 250μM and 500μM achieved similar efficacy. The synergistic effect was dose-dependent in concurrent treatment and post-treatment protocols. On the contrary, dexamethasone addition did not result to significant increase of the anti-proliferative effect of the regimen, either before, concurrently or following cisplatin treatment. Overall, the earlier the BrTaur co-administration started, the better its synergistic effect with cisplatin on CarB cell growth inhibition. Among the three treatment schemes, the inhibitory effect of cisplatin on cell growth was less potent in the post-treatment protocol; however higher doses of BrTaur were able to compensate for the delay of initiation of BrTaur co-administration (Fig 5f, fourth column).

DISCUSSION

Analogous to the microbes that develop a plethora of strategies to eventually become resistant to antibiotics, cancer cells invent several strategies to impair GR and overcome GC-sensitivity.[2] In several cases, a singleton cause of impairment cannot be identified, because GC-resistance is rather multifactorial and attributed to orchestrated inactivation of several GR-controlled pathways.[2,7,8] In this context, trying to identify impaired GR pathway(s) underlying GC-resistance and develop *de novo* a druggable molecule to restore responsiveness poses as a herculean task. A different approach to bypass GC-unresponsiveness of cancer cells would be to reposition alternatives from the pharmaceutical arsenal that are either approved or in late-stages of clinical trials for other GC-refractory inflammatory conditions. Given the emerging commonalities between inflammation and cancer, those alternatives might pose as latent substitutes of GCs' antiproliferative effect, awaiting in a "diamond-in-a-rough" state to be revisited in the context of cancer. This might decrease the pressure for natural selection of cancer cells that overcome the GC antiproliferative effects by deactivating their GR receptor and/or the GR-mediated pathways, the same way that prudent use of antibiotics or use of interchangeable antibiotics prevents the development of antibiotic-resistant microbe strains. Using GC-substitutes before ending-up prescribing GCs would also enable clinical oncologists to reserve the GC-based therapeutic options as a last-resort for aggressive tumors, without risking a possible induction of GC-resistance in earlier tumor stages.

To test this hypothesis we considered the mouse skin carcinogenesis system as our basal screening tool kit. Overall, the characteristics of our study system in terms of GC-sensitivity/GC-resistance and the underlying GR status are summarized in Table II. The model includes a GC-sensitive cell line C5N which retains a functional GR and can be used as the positive, comparative screening control cell line of the panel. The rest of the cell lines

represent GC-resistant papillomas, squamous and spindle cells. In terms of localization, we additionally observed a GR accumulation in the nucleus during transition from squamous-to-spindle stages, resulting to a mixed cytoplasmic and nuclear signal in spindle cells. This unexpected and previously unreported finding, which was reconfirmed in *in vivo* mouse spindle skin cancer tumors, indicates the possible existence of an heterogenous population of GR isoforms and/or variants, some of which may have dominant negative function to the typical full-length isoforms. From the pathology point of view, this means that GR nuclear localization may not be a positive clinical indication for GC-responsiveness, as originally had been suggested [37], especially given the fact that several dominant negative GR isoforms or splice variants that antagonize functional, full-length GR, and cause GC-unresponsiveness are also localized in the nucleus.[1,2]These issues will be clarified in future studies.

BrTaur, our first study case to be checked with our system, presents overlapping characteristics with GCs and is topically used in skin inflammatory conditions, such as acne vulgaris, instead of steroids [13]. It is well-tolerated and presents insignificant side-effects.[13,15] BrTaur surrogated for the antiproliferative effect on GC-sensitive and GC-resistant cells, thus providing the first evidence for its potential to be used interchangeably to GCs in the context of cancer, in the same concept they are currently clinically used interchangeably to GCs in the context of chronic inflammations and microbial infections. The fact that BrTaur efficiently synergizes with cisplatin to inhibit growth of GC-resistant cells further highlights its GC-mimicking therapeutic effect. The antiproliferative effect is strongly linked to the bromine moiety of the bromotaurine molecule and is mediated by inhibition of cell cycle in GC-resistant cells. The ability of BrTaur to produce a more consistent anticancer effect than taurine could be explained by the fact that the former is the oxidizing form, while the latter is the maternal, reservoir substance, considered as a pro-drug. In detail, taurine is retained in several tissues, primarily in liver, and is recruited in tissues undergoing oxidative stress by topically-produced HOCl and HOBr to finally be oxidized to its effective taurine haloamine derivatives. These scavenge the toxicity of the excess HOCl and HOBr and pick up the torch of immunologic responses at the lesion sites, preventing inflammation and exerting anti-microbial and oxidizing properties[13]. In this respect, the inconsistent efficacy of taurine versus N-bromotaurine on the different cell lines of the mouse carcinogenesis system may be due to fluctuated micro-concentrations of HOBr in each different cell line milieu, thus resulting to corresponding fluctuations in the concentration of the active BrTaur finally being formed.

It should be noted that overproliferation in our system, as in actual tumors, is associated with deregulation of several main pathways in addition to GR transactivation impairment.[19] These pathways, such as the ERα and AP-1 oncogenic pathways, crosstalk with GR since they are antagonized by its transrepression mode of action. Their progressive overactivation towards the aggressive stages in our system[22,26,38] implies a dysfunctional GR

transrepression mode additionally to the demonstrated impairment of GR transactivation mode. Furthermore, the mixed cytoplasmic and nuclear signal detected in aggressive stages of skin cancer indicates the possible existence of a heterogeneous population of GR isoforms and/or variants, some of which may have dominant negative function to the typical full-length isoforms. This could be an additional reason for GR's inability to transactivate its targets and, thus, to subsequently mediate the antiproliferative effects of GCs in GC-resistant cells. This would mean that multiple factors causing GC-resistance are possibly accumulating towards the most aggressive stages. Therefore, our system must not be seen as a model dedicated to the study of a single GC-resistance cause. Rather, it should be cautiously used as a tool kit for performing preliminary screenings in order to discriminate the alternative agents with no antiproliferative action from the ones with the potential to restore antiproliferative response in GC-resistant cells.

Notably, although skin cancers are primarily associated with impairment of the GR/GC axis, they are not treated with GCs. Thus our model is not proposed as a means to spot GC-substitutes against this cancer type. However, it is the demonstrated ability of this skin cancer-based artificial model to produce results that are extrapolated to several other types of epithelial cancers[21], including the ones that are commonly treated with GCs, that gives this model an added value as an emerging generalized screening tool kit for identifying GC-substitutes. Using this basal screening tool, substances that are suspected, based on medical experience in the clinic, to have overlapping profiles with GCs could be confirmed as GC-substitutes before being repurposed for the management of cancer patients. Based on this screening tool, investigational N-bromotaurine was shown to act as a GC-substitute, while its effects where reproduced in cancer types that are commonly treated with GCs, such as the GC-resistant human breast and prostate cancer cell lines. Further confirmation of this anti-tumor effect in experimental animals in future studies could accelerate subsequent repositioning of BrTaur for the management of clinical cancer patients.

The point our approach highlights is that already-in-clinical-use compounds, able to consistently induce an antiproliferative phenotype in GC-resistant cells, even via molecular circuits which are currently as unknown to molecular biologists, as are unfamiliar to the GC-resistant cancer cells themselves, warrant to be unveiled and considered as possible GC-substitute therapeutic solutions. Analogous with what happens with newer generation antibiotics that catch the microbes unawares, the success of such compounds to startle cancer cells towards an antiproliferative direction may rely to their possible ability to act through pathways that the cancer cells have never been called to deactivate before in order to become aggressive. Such GC-substitutes may have the clinical potential to prolong disease free-survival, reduce tumor size, delay progression, mitigate side-effects and improve quality of life, in combination with well-established drugs, thus complying to the FDA's

established guidelines on cancer clinical trial end-points. (http://www.fda.gov/downloads/Drugs/Guidances/ucm071590.pdf).

Last but not least, burning issues regarding BrTaur mechanism of action are awaiting to be thoroughly addressed in future studies. Potential ways for its systemic administration in the context of cancer therapeutics should also be explored. Anti-inflammatory effects of BrTaur are mediated by modification of the IkappaBalpha, an NF-kappaB inhibitor[39]. This interaction could also underlie BrTaur antiproliferative effects on GC-resistant cells, given that NF-kappaB stands in the cross-roads between inflammation and cancer[40] and is a crucial effector of GR-mediated tumor-suppressor effects[2]. Given the commonalities shared between anti-inflammatory and anti-cancer pathways, BrTaur may, at least in part, exert its anti-cancer action, in addition to its well-known anti-inflammatory properties[13] by targeting common networks underlying both pathological entities[17,18]. Comprehensive, high-throughput analyses of the molecular and cellular changes and the transcriptional programs alterations triggered upon BrTaur treatment using state-of-the-art, multiomics approaches are anticipated to shed more light on this issue in the future. The full range of BrTaur functions might extend beyond interfering with GR-mediated pathways, and is currently under investigation. The role of BrTaur in cancer emerges as a subject of fruitful research and poses as a mysterious “black box”, the decoding of which might pave the way for next generation therapeutics.

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FIGURE LEGENDS

Figure 1: Estimation of responsiveness of cell lines of the mouse skin carcinogenesis model to GCs (dexamethasone) by crystal violet assays. **(a)** In the representative time point of 48h, the number of cells drastically decreases proportional to dexamethasone concentration in comparison with the untreated cells in the C5N cell line (expressed as ratio to untreated cells). In contrast, there is no change in cell numbers of dexamethasone-treated P6, B9, A5 and CarB cells compared to their corresponding untreated controls, in any of the concentrations tested. **(b)** The profile of the antiproliferative action of GCs on the GC-sensitive C5N cells over time, for each of the tested dexamethasone concentrations in comparison to a selected GC-resistant cell line P6. The experiments were performed in triplicates.

Figure 2: GR is translocated in nucleus during transition from squamous to spindle stage of skin carcinogenesis. **(a)** GR protein expression levels of total (tGR), cytoplasmic (cGR) and nuclear (nGR) extracts of the mouse skin carcinogenesis model. **(b)** Western blot results indicate a switch in the cytoplasmic-to-nuclear GR ratio (cGR-to-nGR) during transition from squamous to spindle stage. **(c)** Immunocytochemistry analysis of the panel of mouse skin carcinogenesis model with anti-GR antibody revealed cytoplasmic staining in C5N, P6 and B9 cells, and mixed cytoplasmic and nuclear staining in A5 and CarB cells. The black arrows indicate GR-stained nuclei. These immunocytochemical patterns were rather uniform in the above mentioned cell lines. **(d)** The immunofluorescence GR signal is cytoplasmic in the squamous B9 cells, but nuclear in the spindle A5 cells. **(e)** Immunohistochemistry using anti-GR antibody in paraffin-embedded tissues isolated from the papilloma, squamous and spindle stage of the same chemically-induced tumor in one mouse revealed higher number of GR-stained nuclei in spindle cells in comparison with the corresponding papilloma and squamous stage. Each figure represents a 200x magnitude, whereas the upper-right boxes in each figure represent a 400x magnitude. The black boxes represent the magnified area. The black arrows indicate GR-stained nuclei.

Figure 3: GR is DNA binding-competent but transactivation-incompetent in GC-resistant cell lines of the mouse skin carcinogenesis model. **(a)** Electrophoretic mobility shift assay in the GC-responsive C5N revealed binding activity of GR to glucocorticoid responsive elements (GRE) only upon dexamethasone (C5N+dex) treatment. **(b)** EMSA assay for GR using nuclear extracts demonstrated binding activity of GR to glucocorticoid responsive elements (GRE) of the GC-unresponsive P6, B9, A5 and CarB cells in the absence of dexamethasone signal. DNA binding increases directly proportional to the demonstrated nuclear GR protein

levels towards more aggressive cell lines. Specificity of the EMSA reaction is confirmed by supershift reactions in A5 (lane 6) cells using anti-GR antibody. **(c)** Luciferase assays in the GC-sensitive and the GC-resistant cells of the mouse skin carcinogenesis model co-transfected with the 17m-GRE-G-Luc, in dexamethasone absence (-dex) or presence (+dex). Luciferase expression is significantly induced (One asterisk (*) denotes $p < 0.05$) only in the GC-sensitive C5N cells upon dexamethasone treatment. Error bars represent the standard error of the mean. Two-tailed p values are are derived from independent t-tests. Three biological replicates were available for each experiment. **(d)** Box plots demonstrating the transcription induction of GILZ under dexamethasone treatment in in the GC-sensitive and the GC-resistant cell lines before (-dex) and after (+dex) dexamethasone treatment, estimated by qPCR. The relative quantification (RQ) values in $\log_{10}$ are shown. For each cell line, the untreated control was used as calibrator. The housekeeping gene beta-2 microglobulin was used as an internal control of mRNA Q-PCR expression. The experiments were performed in triplicates. **(e)** Same as 3d, for $p57^{KIP2}$ mRNA levels. In both genes, significant induction is only observed in C5N cells (indicated by star). RQ: relative quantification value.

Figure 4: Estimation of the antiproliferative response of the GC-sensitive and the GC-resistant cell lines to N-bromotaurine by proliferation assays. **(a)** In the representative time point of 48h, the number of cells drastically decreases in comparison with the corresponding untreated control cells for all cell lines of the study model (expressed as ratio to untreated cells). **(b)** Comparative treatment of the cells with the maternal substance taurine did not affect mouse skin cancer cell proliferation in a significant and consistent manner, implying that bromination of the taurine is the crucial factor for the consistent antiproliferative effect on cells. **(c)** The profile of the antiproliferative action of N-bromotaurine on the GC-resistant GC-resistant prostate cancer PC3. **(d)** Same as 4c for the human GC-resistant prostate cancer MDA-MB-231. The experiments were performed in triplicates.

Figure 5: Comparison of the antiproliferative response of the aggressive GC-resistant cells to GCs versus N-bromotaurine. **(a)** proliferation assays on CarB cells treated with dexamethasone reveal lack of significant antiproliferative response of CarB upon addition of GCs over time, as evidenced by the OD measurements. **(b)** In contrast, proliferation assays on CarB cells treated with N-bromotaurine instead of dexamethasone reveal restoration of antiproliferative response of CarB cells by N-bromotaurine over time. The restoration of antiproliferative response is statistically significant in the 250μM concentration, after 48 and 72 hours of N-bromotaurine treatment. Three independent experiments were performed, each

in triplicate. The means+SEM values are shown in the graphs. **(c)** FACS analysis of N-bromotaurine-treated CarB cells (CarB + BrTaur) compared with dexamethasone-treated CarB cells (CarB + dex) and untreated CarB cells (CarB). BrTaur, at concentrations of 125μM and 250μM, increased the number of cells in G0/G1 phase; whereas at the high concentration of 500μM, BrTaur drastically decreased both G0/G1 and G2/M phases. Dexamethasone, at 10^{-7}M concentration failed to significantly affect the number of cells in G1/G0 phase. Corresponding summary graphs for each phase of the cell cycle (means±SEM from 3 independent FACS analyses measurements) derived by comparison of untreated CarB cells each of the dexamethasone-treated, 125μM BrTaur-treated, 250μM BrTaur-treated and 500μM BrTaur-treated CarB cells. **(d-f)** 250μM BrTaur-treated and 500μM BrTaur-treated CarB cells synergized efficiently with 2.9 cisplatin (cis) after 48hours of BrTaur plus cisplatin combination therapy as **(d)** pre-treament (BrTaur + cis), **(e)** concurrent treatment (cis/BrTaur) or **(f)** post-treatment (cis + BrTaur). Results are flagged with no asterisk when p-value is more than 0.05, and with two asterisks when the p-value is less than 0.001.(See text for details on treatment schemes of 5d-f).

Table I

Table I: Primers used for Q-PCR reactions

Q-PCR amplicon	Primers	Primer sequence	Annealing temperature
$p57^{KIP2}$	F	5- CCTCTTCGGGCCTGTAGAC -3	59°C
	R	5- CACCGTCTCGCGGTAGAA -3	
GILZ	F	5-TAACACTGTCTGGTAACGATGTTAA-3	
	R	5-TAACACTGTCTGGTAACGATGTTAA-3	
β2-microglobulin	F	5- GCATGGCTCGCTCGGTGAC -3	
	R	5- GGCGTATGTATCAGTCTCAGTG-3	

Table II

Table II: Mouse skin carcinogenesis model features, in correlation with aspects of GR status in each cell line of the model.

Model features / GR features	**INITIATION**	**PROMOTION**		**PROGRESSION**	
	C5N	P6	B9	A5	CarB
	GC-sensitive	GC-resistant	GC-resistant	GC-resistant	GC-resistant
EXPRESSION					
Nuclear GR levels	+	+	++	++++	++++
LOCALIZATION					
GR localization	cyt	cyt	cyt	nucl+cyt	nucl+cyt
DNA BINDING					
binding to GRE elements	+	+	++	++++	++++
TRANSACTIVATION MODE					
Transactivation of GC-responsive antiproliferative GRE-containing targets	+	-	-	-	-
Mutation in GR transactivation domains (exons 2, 5 and 9)	NO	NO	NO	NO	NO
TRANSREPRESSION MODE					
Active AP-1 pathway[38]	+	++	+++	++++	++++
Active ERα pathway[22]	+	++	++++	++++	++++

cyt: cytoplasmic; nucl: nuclear

The bold line of the table represents the threshold between promotion and progression stages in the mouse skin carcinogenesis system.

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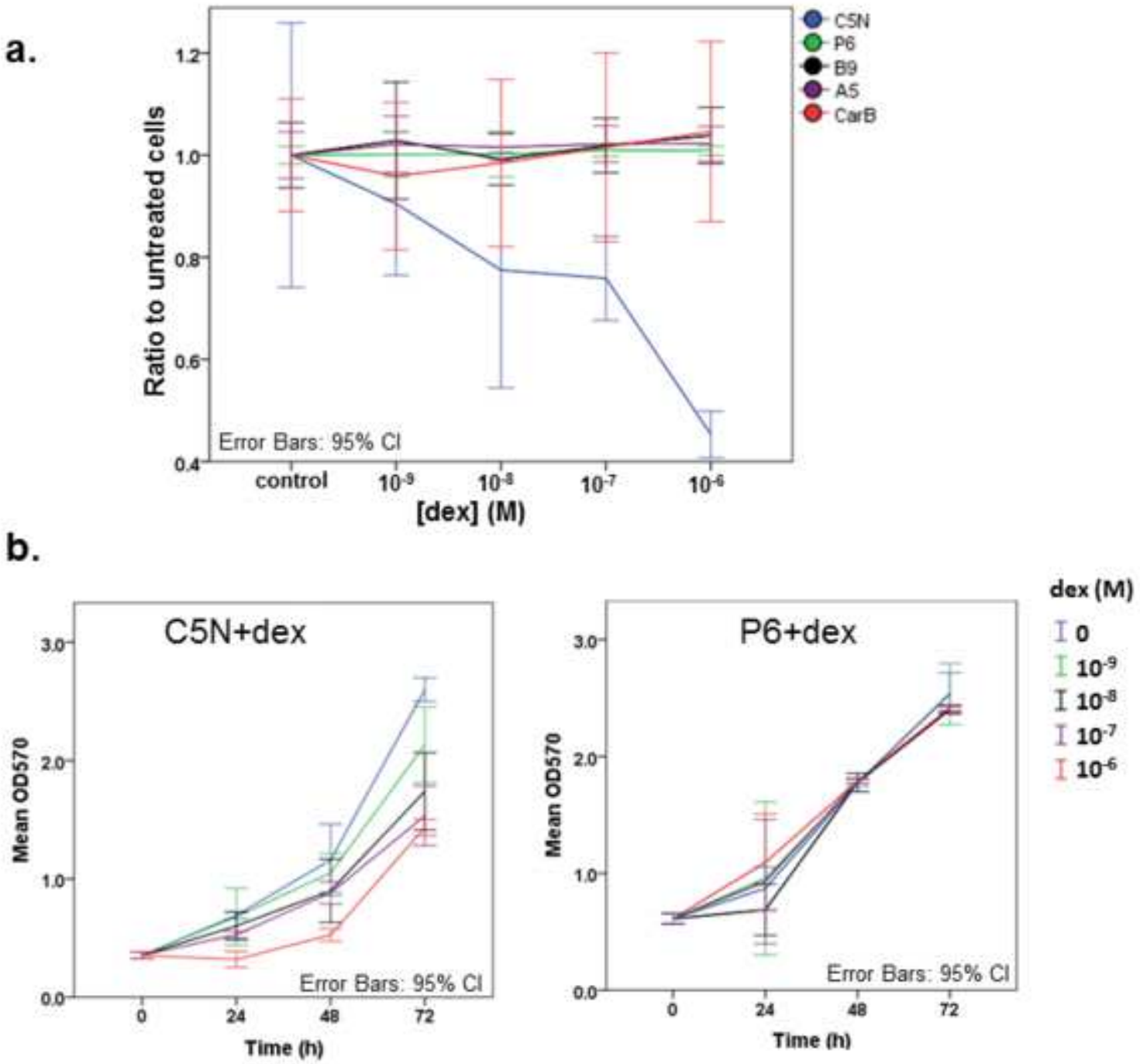

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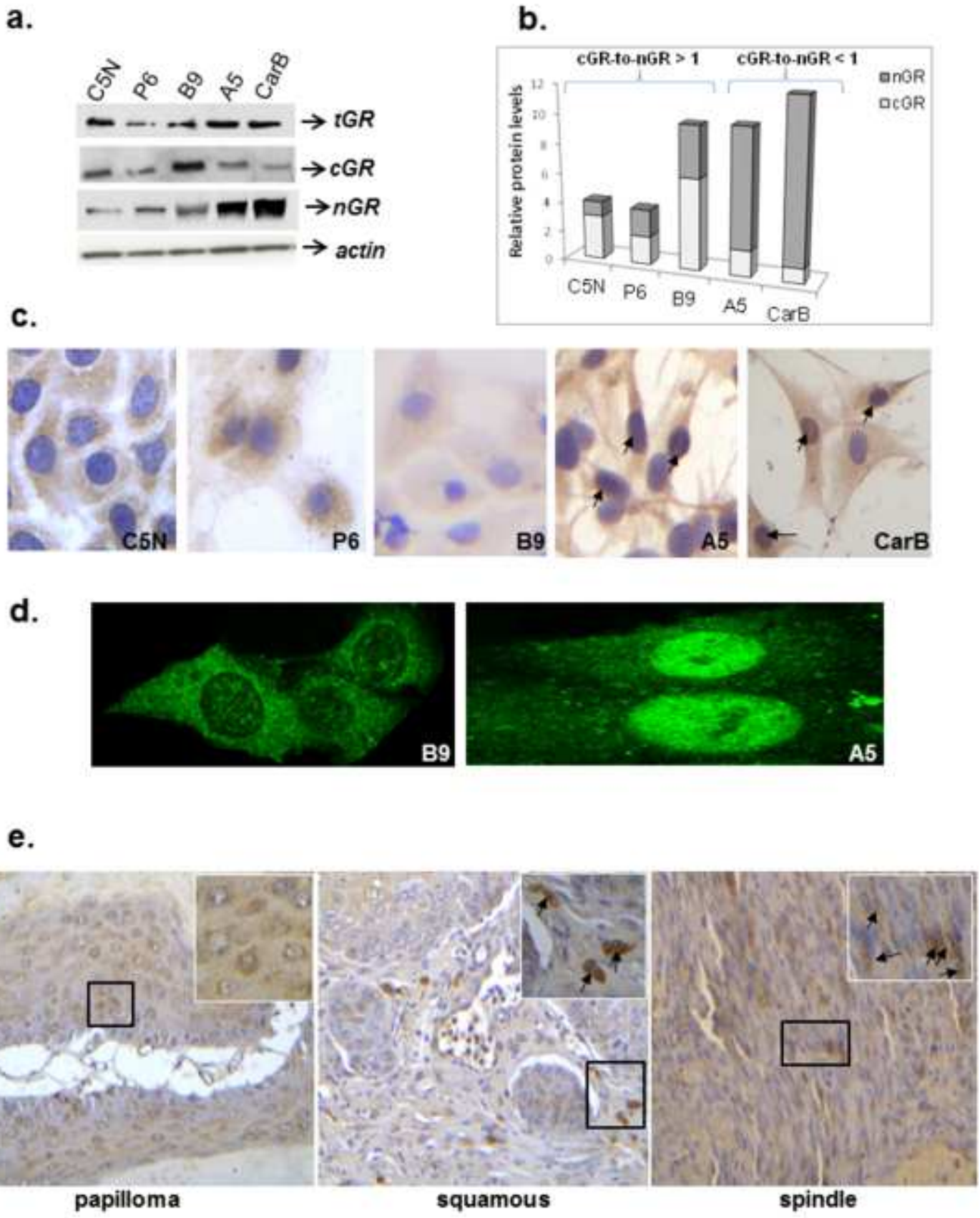

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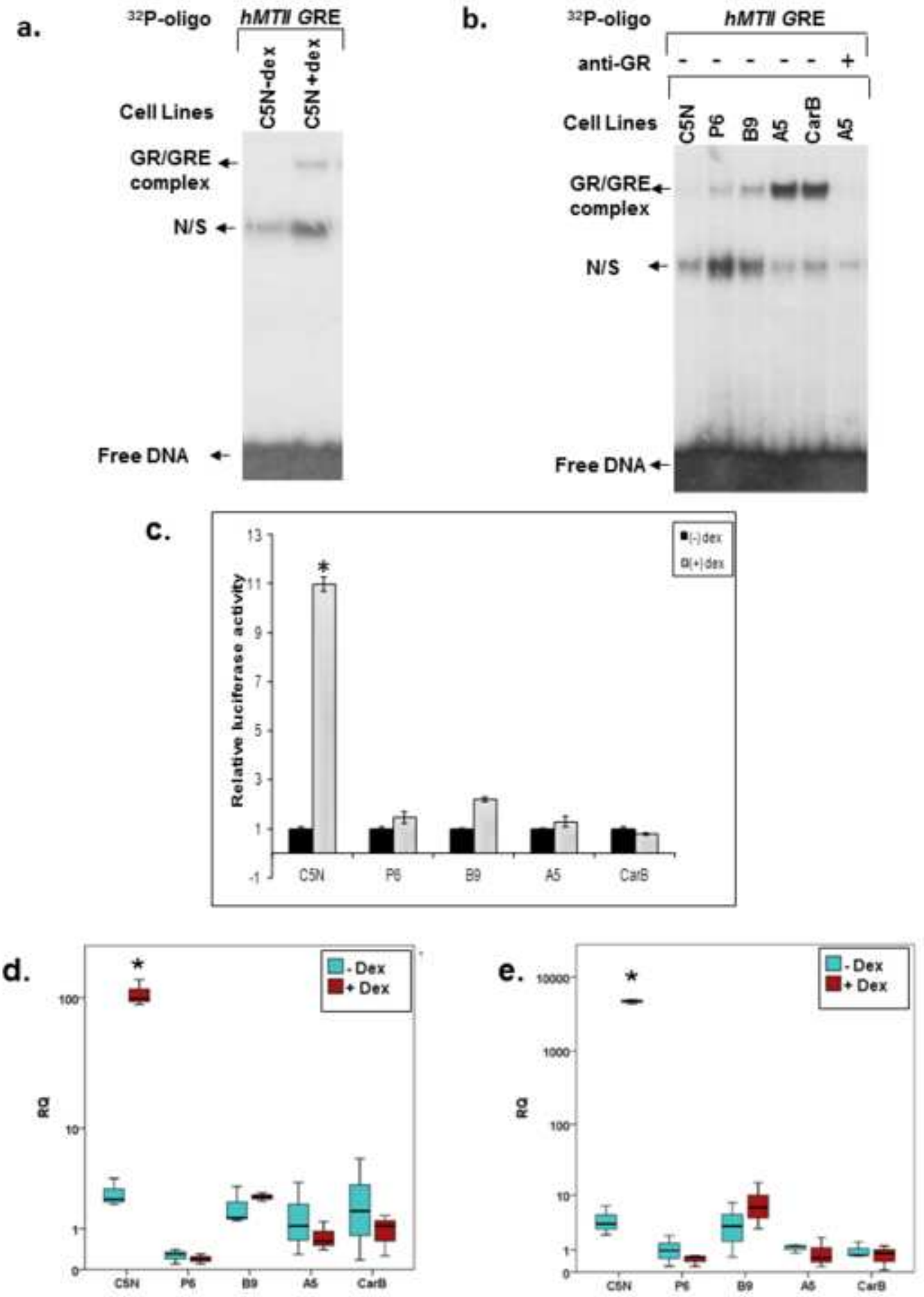

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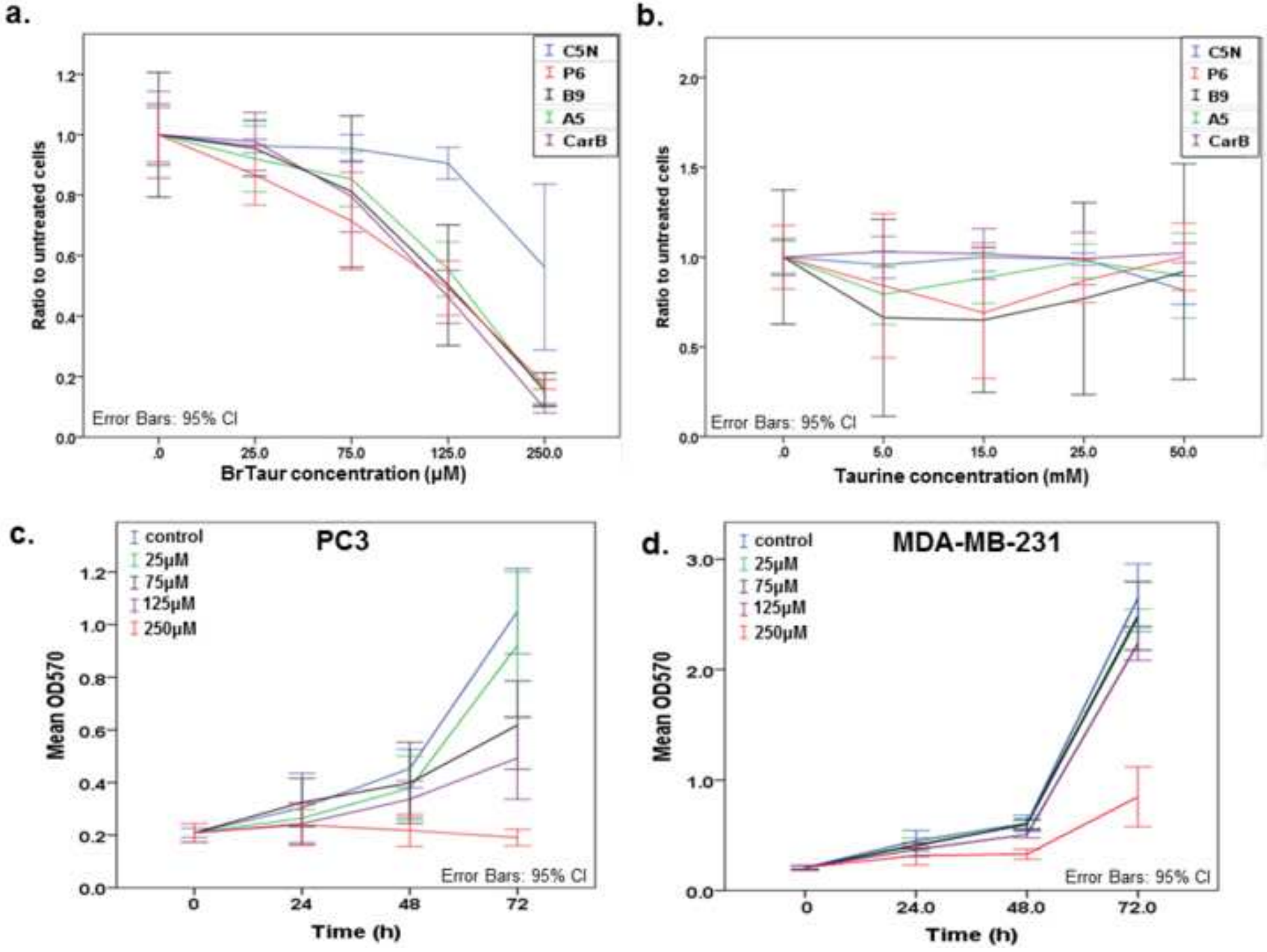

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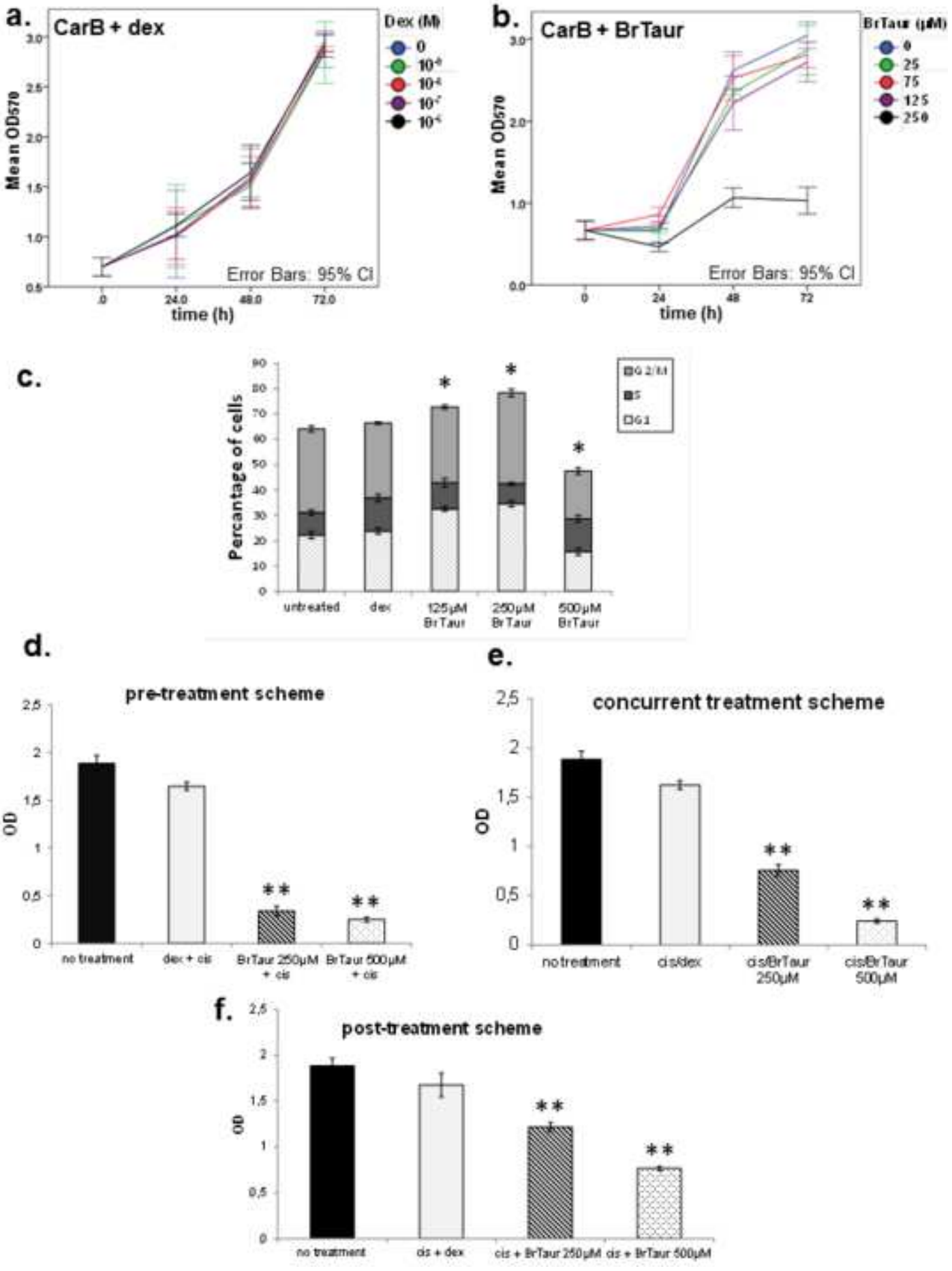

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