Cancer Letters 571 (2023) 216338

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

The CAIX inhibitor SLC-0111 exerts anti-cancer activity on gastric cancer cell lines and resensitizes resistant cells to 5-Fluorouracil, taxane-derived, and platinum-based drugs

Elena Andreucci [a,1], Alessio Biagioni [a,1], Sara Peri [b], Giampaolo Versienti [a], Fabio Cianchi [b,c,**], Fabio Staderini [b,c], Lorenzo Antonuzzo [b,d], Claudiu T. Supuran [e], Erika Olivo [f], Elisa Pasqualini [f], Luca Messerini [b], Daniela Massi [f], Matteo Lulli [a], Jessica Ruzzolini [a], Silvia Peppicelli [a], Francesca Bianchini [a], Nicola Schiavone [a], Lido Calorini [a], Lucia Magnelli [a,*], Laura Papucci [a]

[a] *Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Viale GC Morgagni 50, 50134, Firenze, Italy*
[b] *Department of Experimental and Clinical Medicine, University of Florence, Largo Brambilla 3, 50134, Firenze, Italy*
[c] *Unit of Digestive Surgery, Careggi University Hospital, Largo Brambilla 3, 50134, Firenze, Italy*
[d] *Clinical Oncology Unit, Careggi University Hospital, Largo Brambilla 3, 50134, Firenze, Italy*
[e] *Department of NEUROFARBA, University of Florence, Via Ugo Schiff 6, 50019, Sesto Fiorentino, Italy*
[f] *Department of Health Sciences, University of Florence, Viale Pieraccini 6, 50139, Firenze, Italy*

A R T I C L E I N F O

Keywords:
Gastric cancer
Carbonic anhydrase IX
Perioperative chemotherapy
Chemoresistance

A B S T R A C T

Gastric cancer (GC) is the fifth most frequent malignancy and the fourth leading cause of worldwide cancer-related death. Despite the usage of multimodal perioperative chemotherapy (pCT), GC progressively gains chemoresistance, thereby, the identification of suitable targets to overcome drug resistance is fundamental. Amongst the potential biomarkers, carbonic anhydrase IX (CAIX) - associated with a poor prognosis of several solid cancers - has gained the most attention. In a cohort of GC patients who received perioperative FLOT (i.e., Leucovorin, 5-Fluouracil, Docetaxel, and Oxaliplatin) or FOLFOX (i.e., Leucovorin, 5-Fluouracil, and Oxaliplatin), non-responder patients showed an increased expression of tumor CAIX compared to responder group. Moreover, GC cell lines induced to be resistant to 5-Fluouracil, Paclitaxel, Cisplatin, or the combination of 5-Fluorouracil, Oxaliplatin, and Docetaxel, overexpressed CAIX compared to the control. Accordingly, CAIX-high-expressing GC cells showed increased therapy resistance compared to low-expressing cells. Notably, SLC0111 significantly improved the therapy response of both wild-type and resistant GC cells.

Overall, these data suggest a correlation between CAIX and GC drug resistance highlighting the potential of SLC-0111 in re-sensitizing GC cells to pCT.

1. Introduction

Gastric cancer (GC) represents the fifth most frequently diagnosed cancer accounting for more than 1,000,000 new cases per year and about 760,000 deaths worldwide [1]. Although surgical resection with D2 lymphadenectomy remains the mainstay of curative therapy, the patient's prognosis remains overall poor due to the high incidence of advanced and inoperable disease at diagnosis, and the high percentage of progression after curative surgery. For this reason, multimodal approaches with perioperative and adjuvant chemotherapy and surgery have been developed [2].

Chemotherapy (CT) before surgery increases the chance for curative resection, sterilizes micro-metastasis, and allows an *in vivo* response assessment to further CT. Based on randomized controlled trials, perioperative CT became the standard of care for patients with locally advanced GC in Europe [2]. Recently, the FLOT regimen (5-FU, Folinic acid, Oxaliplatin, and Docetaxel) obtained better pathological response rates and higher R0 resection rates compared with the ECF regimen (*i.e.*, Epirubicin, Cisplatin, and 5-FU) in a prospective randomized controlled

* Corresponding author.
** Corresponding author. Department of Experimental and Clinical Medicine, University of Florence, Largo Brambilla 3, 50134, Firenze, Italy.
E-mail addresses: fabio.cianchi@unifi.it (F. Cianchi), lucia.magnelli@unifi.it (L. Magnelli).
[1] EA and AB contributed equally.

https://doi.org/10.1016/j.canlet.2023.216338
Received 27 April 2023; Received in revised form 4 August 2023; Accepted 4 August 2023
Available online 6 August 2023
0304-3835/© 2023 Published by Elsevier B.V.

Abbreviations

5-FU	5-fluorouracil
CAIX	Carbonic Anhydrase IX
CIS	cisplatin
GC	gastric cancer
pCT	perioperative chemotherapy
PTX	paclitaxel
TRG	tumor regression grade

trial [3]. Furthermore, FLOT resulted in a better overall survival (hazard ratio [HR] 0·77, 95% CI 0·63–0·94; median overall survival 50 months [38·33 to not reached] vs 35 months [27·35–46·26]) thus becoming the recommended standard of care for patients with respectable GC who can tolerate a perioperative three-drug combination regimen [4]. Nevertheless, the development of drug resistance and the subsequent tumor relapse represents the main cause of death in patients who undergo systemic CT. Thereby, the identification of biomarkers of GC resistance to preoperative CT is mandatory. Amongst the potential biomarkers, the carbonic anhydrase IX (CAIX) has gained the most attention, since its overexpression is associated with a poor prognosis of several solid cancers, including lung, pancreas, breast, and cervical cancer [5]. CAIX maintains a favorable intracellular pH for tumor cell survival and growth and functions as a chemokinetic factor that drives migration and invasion by both catalytic and non-catalytic mechanisms, which fosters the formation of metastasis [6,7]. Further, CAIX was found as a key mediator of drug resistance [6]. The role of CAIX as a critical marker in the molecular biology of GC has not been yet elucidated. Interestingly, primary gastric tumors and GC cell lines show lower CAIX levels compared to normal mucosa unlike most other cancer types, in which CAIX is overexpressed in comparison with healthy tissues; a correlation with shorter postoperative survival has been observed in those patients with gastric tumors that retain and/or re-express CAIX [8]. In addition, the CAIX inhibitors such as the ureido-benzene-sulphonamide SLC-0111 are currently giving promising results as sensitizers towards conventional chemotherapeutic agents in several pre-clinical settings [9–11]. Notably, SLC-0111 is currently under phase Ib/II clinical trial evaluation for metastatic pancreatic ductal cancer treatment (https://clinicaltrials.gov/ct2/show/NCT03450018).

Here we demonstrated, for the first time, that GC patients who did not respond to perioperative FLOT/FOLFOX treatment expressed significantly higher levels of CAIX compared to the responder patients' group. In parallel, we demonstrated *in vitro* that experimentally induced chemoresistant GC cell lines overexpress CAIX in comparison with the relative wild-type/sensitive counterpart. We hypothesized that this phenomenon is related to the glycolytic addiction of chemoresistant GC cell lines. After the isolation of the high- and low-expressing CAIX cell subpopulations and the molecular forcing of CAIX expression in AGS cells, we proved a close association between CAIX activity and the drug-resistant phenotype. Importantly, the CAIX inhibitor SLC-0111 could restore GC cell response to standard chemotherapeutic agents, so it could be proposed as an intriguing complementary therapeutic option to overcome drug resistance.

2. Materials and methods

2.1. Patients' selection and retrospective analysis

Twenty-six GC patients who underwent pre-operative therapy followed by gastrectomy at the Azienda Ospedaliero Universitaria Careggi (AOUC) in Florence were classified as responder and non-responder, depending on the tumor regression grade (TRG 1–2 for responder versus TRG 3–5 for non-responder) established by the pathologists' board basing on Mandard score. Three patients of the responder group were excluded by the analysis as showing a complete tumor regression following pre-operative therapy (TRG 1), and therefore tumor CAIX expression could not be assessed. The use of Formalin-fixed Paraffin-embedded (FFPE) sections of human samples was approved by the Local Ethics Committee of University Hospital, Azienda Ospedaliero-Universitaria Careggi, Florence, Italy (n.10,780), according to the Helsinki Declaration, and informed consent was obtained from each alive patient enrolled in the study.

2.2. Immunohistochemistry

Formalin-fixed Paraffin-embedded tumor biopsies were analyzed by immunohistochemistry (IHC) to detect the CAIX levels. Immunohistochemistry was performed in the Ventana Discovery Ultra Immunostainer. Representative FFPE whole tumor sections 3 μm thick were deparaffinized in EZ prep (#950-102; Ventana), and antigen retrieval was achieved by incubation with cell-conditioning solution 1 (CC1) (#950-124; Ventana). Sections were incubated with *anti*-CAIX primary antibody (dilution 1:80, mouse monoclonal, abcam ab107257, Cambridge, UK). The signal was developed with UltraMap anti-mouse HRP. The bound antibody was visualized using Chromomap DAB (#760-159, ready to use, Ventana Medical Systems, Tucson, AZ, USA). At last, the sections were counterstained with Hematoxylin II (#790–2208, ready to use, Ventana Medical Systems, Tucson, AZ, USA). Normal gastric tissue was used as a positive control. Negative control was performed by substituting the primary antibody with Mouse IgG1 kappa Isotype Control Clone P3.6.2.8.1, dilution 1:80 (Invitrogen, Catalog # 14-4714-82). The negative and positive control sections were treated in parallel with the samples in the same run. The immunohistochemical expression of CAIX was evaluated according to Nakamura and colleagues [12]. CAIX membrane labeling was assessed as high or low combining the percentage and the intensity of staining. Scoring for the percentage of stained cells was as follows: 0, no cells staining positive; 1, <1% positive cells; 2, 1%–10% positive cells; 3, 10%–33% positive cells; 4, 33%–66% positive cells; and 5, >66% positive cells. Scoring for the intensity was as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. Tumors with a total score of >6 were considered to have high expression of CAIX [12].

2.3. Cell Culture and treatments

AGS GC cells [13] were purchased from ATCC (CRL-1739) and the 23,132/87 cell line [14] (hereafter referred to as ACC-201) was provided by the Leibniz Institute (DSMZ-German Collection of Microorganisms and Cell Cultures). AGS and ACC-201 cells were maintained in F12K medium (Corning, Milano, Italy) and RPMI1640 (Euroclone, Milano, Italy) respectively, both supplemented with 10% FBS (a dedicated lot from Euroclone, Milan, Italy) and 1% L-glutamine (Euroclone). AGS cell line exhibits typical epithelial morphology and was derived by 54 years old female patient's gastric adenocarcinoma whilst ACC-201 was established from a 72-year-old man gastric adenocarcinoma and has the tendency to grow in clusters. Both lines were derived from Caucasian patients. Cells were tested every two weeks for Mycoplasma by PCR using two universal primers (MGSO and GPO1) [15]. 5-Fluorouracil (5-FU, F6627), Paclitaxel (PTX, T7402), and Cisplatin (CIS, P4394) were purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy) while Oxaliplatin (HY-17371) and Docetaxel (HY–B0011) from MedChemExpress (distributed by DBA, Milan, Italy). The CAIX inhibitor SLC-0111 was provided by Professor Claudiu T. Supuran (NEUROFARBA Department, University of Florence, Italy). GC cells resistant to 5-FU (5-FUr), Paclitaxel (PTXr), Cisplatin (CISr), and the combination of the anti-cancer agents 5-FU, Docetaxel, and Oxaliplatin used in the FLOT regimen (FLOTr) were generated following the "high-level laboratory models" reported by McDermott and colleagues [16]. AGS chemoresistant cell lines were previously characterized and published by Peri

S. and colleagues [13,17].

2.4. IC50 assay

5×10^3/well GC cells were plated in a 96-well plate. After 24 h the culture medium was replaced and cells were treated with scalar dilutions of drugs. 72 h later, cells were incubated for 2 h at 37 °C in the dark with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in medium without phenol red. MTT was removed and cells were lysed in 100 μl DMSO. Absorbance was recorded at 595 nm with an automatic plate reader (Bio-Rad, Milan, Italy).

2.5. Seahorse

3.0×10^4/well GC cells were seeded onto Seahorse XFe96 microplates and glycolytic metabolism evaluated with the Seahorse XFe96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA) using the Glycolytic Rate Assay Kit (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instruction. All the experiments were performed at 37 °C and normalized via cell protein measure with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

2.6. Lactate

The Lactate Colorimetric Assay Kit (BioVision, purchased from Vinci-Biochem, Florence, Italy) was used according to the manufacturer's instructions to measure lactate production in the conditioned media of GC cells, as previously described [18].

2.7. Gas cromatography mass spectrometry (GC-MS)

GC cells were serum-starved for 6 h and prepared for GC-MS analysis on an Intuvo9000/5977 B MSD (Agilent Technologies, Santa Clara, CA, USA) via selected ion monitoring (SIM) mode MS. Briefly, cells were washed twice with 0,9% NaCl at 4 °C and scraped in 400 μl of cold (−20 °C) 80% methanol in water (containing 1 μg/mL norvaline as internal standard). Samples were sonicated on ice for 5 s for 3 times with a 5 s interval at 70% amplitude, centrifuged at 14,000 rpm, 4 °C for 10 min, and supernatants were collected and lyophilized. Cell pellets were resuspended in 50 μl of 200 mM NaOH, denatured at 96 °C for 15 min, centrifuged at 14,000 rpm for 5 min, and used for establishing the protein concentration per sample for final normalization.

2.8. Flow cytometry analysis

2×10^5 cells GC cells were harvested and collected in flow cytometer tubes, and stained for 1 h at 4 °C in the dark with 1:100 *anti*-CAIX conjugated with Alexa Fluor 488 (sc-365,900 AF488; Santa Cruz Biotechnology, DBA, Milan, Italy). Cells were washed in PBS and analyzed at BD FACSCanto II (BD Biosciences) using the Alexa Fluor 488-conjugated irrelevant IgG to set the gating. To isolate the CAIX-high and CAIX-low-expressing cells, the AGS WT population was sorted at the FACS Melody (BD Bioscience) following CAIX staining as above. Appropriate gates were defined to isolate the 25%-low-expressing cells and the 25%-high-expressing cells.

2.9. CAIX overexpression

AGS cells were transfected via Lipofectamine 3000 (ThermoFisher Scientific, Milan, Italy) with the empty vector or the pRP-EGFP/Neo-CMV > hCA9 overexpression plasmid where CAIX-ORF was in frame with the eGFP and geneticin resistance coding sequences (Vector Builder, China). Following geneticin selection, the CAIX-overexpressing subpopulation was purified at the FACS Melody exploiting the eGFP positivity.

2.10. CAIX silencing

WT and FLOTr AGS and ACC-201 cells were transfected via Lipofectamine 3000 (ThermoFisher Scientific, Milan, Italy) with MISSION siRNA Universal Negative Control or MISSION esiRNA Human CA9 (Sigma-Aldrich, Milan Italy) according to the manufacturer's instructions. MISSION® esiRNA consists of a heterogeneous mixture of endoribonuclease-prepared siRNA that all target the same mRNA sequence, allowing highly-specific and effective gene silencing. Control (siCTRL) and CAIX-silenced (siCAIX) WT and FLOTr AGS and ACC-201 cells were tested for CAIX protein expression by Western blot and flow cytometry, to detect either the total CAIX levels or the plasma membrane CAIX expression 72 h after the transfection.

2.11. Western blot analysis

AGS and ACC-201 GC cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Merck Millipore, Milan, Italy) added with Pierce Protease Inhibitor Tablets (Thermo Fisher Scientific, Milan, Italy) for protein isolation. The protein concentration was measured with Bradford reagent (Merck Millipore, Milan, Italy), and 60 μg of proteins per well in Laemmli buffer were separated on 8%–12% (v/v) SDS-PAGE gel (Thermo Fischer Scientific, Milan, Italy) and transferred to a polyvinylidene difluoride (PVDF) membrane using the iBlot 2 System (Thermo Fischer Scientific, Milan, Italy). Membranes were incubated for 5 min with EveryBlot Blocking Buffer (Bio-Rad, Milan, Italy), and then probed overnight at 4 °C with *anti*-CAIX-AF680 (sc-365,900-AF680, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and *anti*-vinculin (sc-73614, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following 1-h incubation at RT with goat anti-mouse IgG Alexa Fluor 680 antibody (Thermo Fisher Scientific, Milan, Italy), membranes were visualized at the Odyssey Infrared Imaging System (LI-COR® Bioscience, Lincoln, NE, USA).

2.12. In-cell western assay

2.0×10^4 cells/well GC cells were seeded into a 96-well plate, fixed with 4% paraformaldehyde, and permeabilized by PBS 0.1% Triton X-100. After 1 h-blocking with Immobilion Block – FL (Fluorescent Blocker, Millipore, Sigma-Aldrich, Milan Italy), cells were stained for 2 h with the 1:100 *anti*-CAIX (Abcam, Prodotti Gianni, Milan, Italy) and the 1:100 anti-tubulin (Cell Signaling, EuroClone, Milan, Italy) primary antibodies and then for 1 h with 1:1000 goat anti-rabbit and goat anti-mouse secondary antibodies (ThermoFisher Scientific, Milan, Italy). The plate was washed in PBS 0.1% Tween and analyzed at the Odyssey Imager (LI-COR, Bad Homburg, Germany).

2.13. Cell viability

Apoptosis was evaluated using either Annexin V-APC/PI (Immunotools, Germany) as previously described [19] or the Cell Event Caspase 3/7 Green Flow Cytometry assay kit (Life Technologies, Milan, Italy), according to the manufacturer's instructions. Samples were then analyzed at the BD FACSCanto II flow cytometer (BD Bioscience, Milan, Italy).

2.14. Colony formation assay

0.5×10^3/well GC cells were seeded in 6-well plates and treated in the complete medium with the IC50 dose of each chemotherapeutic agent for 7 days to allow colony growth. Colonies were fixed in 3.7% paraformaldehyde and stained with crystal violet solution as previously described [20].

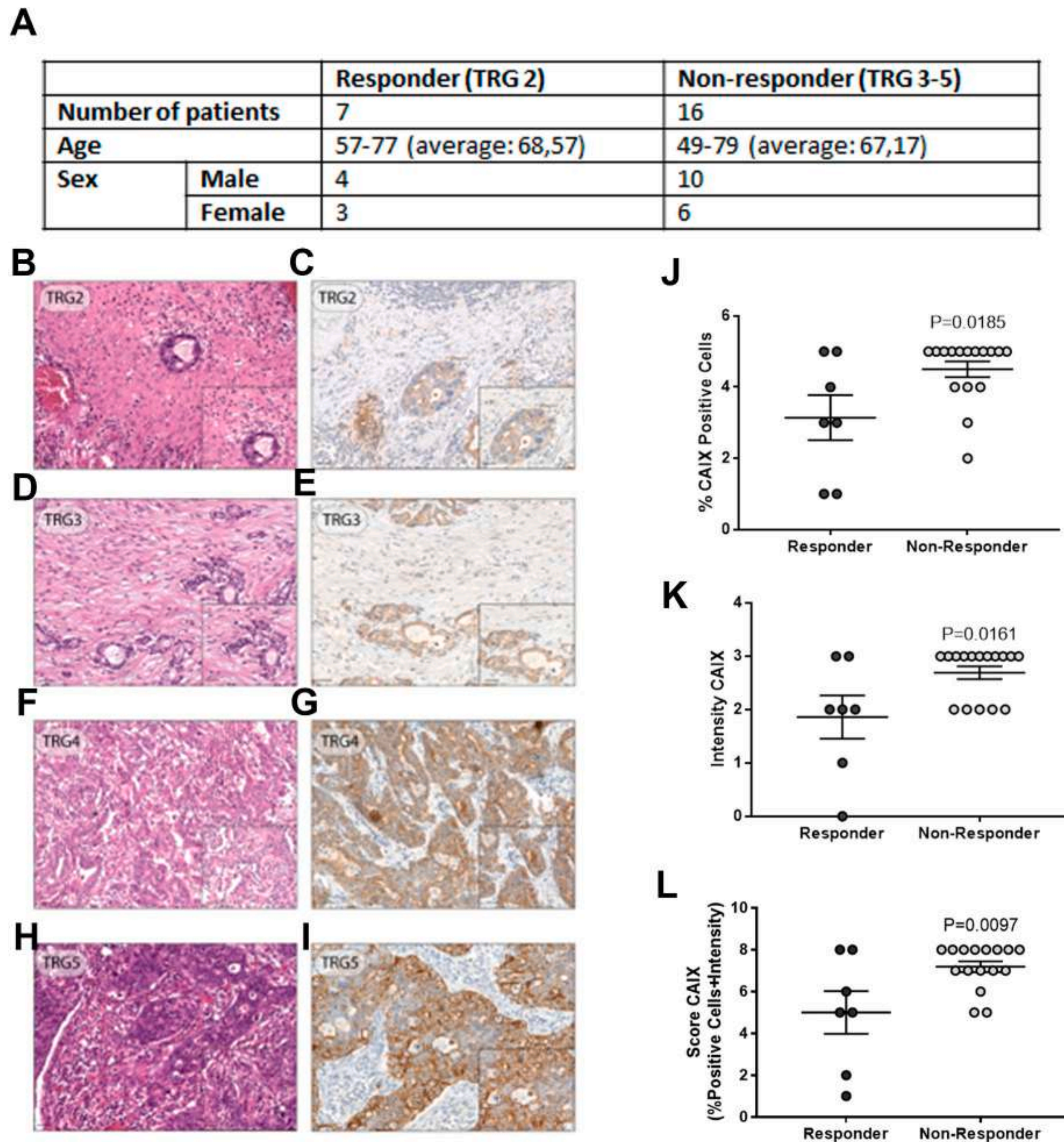
2.15. Statistical analysis

All data were obtained based on at least three independent experiments. Statistical analysis was performed with GraphPad Prism 7 software by *t*-test, one-way analysis of variance (ANOVA), and two-way ANOVA, as specified in each figure legend. Values are presented as mean ± standard deviation (SD). The p values are presented as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results

3.1. Tumor CAIX expression is increased in non-responders compared to responder GC patients who have undergone preoperative FLOT or FOLFOX regimen

Twenty-three responder and non-responder GC patients who underwent perioperative FLOT/FOLFOX therapy (FLOT N = 16, FOLFOX N = 7) (Fig. 1a and Supplementary Table), were grouped based on the TRG value as described in the Material and Methods section. The two groups showed a significant differential tumor expression of CAIX (Fig. 1b–i). In particular, the non-responder group was characterized by a significantly increased percentage, intensity, and score of CAIX



		Responder (TRG 2)	Non-responder (TRG 3-5)
Number of patients		7	16
Age		57-77 (average: 68,57)	49-79 (average: 67,17)
Sex	Male	4	10
	Female	3	6

Fig. 1. CAIX expression in GC tissues with different TRG values. a) Responder and non-responder GC patients to FLOT/FOLFOX regimen. **b-i)** Representative images of TRG2 **(b)** Hematoxylin & Eosin and **(c)** CAIX IHC staining. Representative images of TRG3 **(d)** Hematoxylin & Eosin and **(e)** CAIX IHC staining. Representative images of TRG4 **(f)** Hematoxylin & Eosin and **(g)** CAIX IHC staining. Representative images of TRG5 **(h)** Hematoxylin & Eosin and **(i)** CAIX IHC staining. (Magnification × 20, inset × 40; scale bar 100 μm, 50 μm, respectively). **j-l)** Percentage of positive (**j**), intensity (**k**) and score (**l**) of the CAIX staining in tumor samples of responder and non-responder GC patients affected by gastric adenocarcinoma and subjected to gastrectomy following the perioperative FLOT/FOLFOX therapeutic regimen (T-test).

(Fig. 1j-l).

3.2. Drug-resistant GC cells are characterized by a boosted glycolytic metabolism compared to wild-type cells

Representative pictures of wild-type and drug-resistant AGS and ACC-201 are shown in Fig. 2a. The resistance to each single (5-FU, PTX, or CIS) or combined treatment (FLOT) is reported in Fig. 2b and c for AGS and ACC-201 cells, respectively. The Glyco Rate Assay performed with the Seahorse XFe96 analyzer showed that drug-resistant GC cells are characterized by a boosted glycolytic metabolism (Fig. 3a), accompanied by an increased lactate production (Fig. 3b) and a reduced amount of TCA intermediate metabolites, such as citrate, α-ketoglutarate, and malate, as revealed by the GC-MS analysis (Fig. 3c), compared to the wild-type cells.

3.3. Drug-resistant GC cells showed an increased expression of CAIX compared to wild-type cells

Parallel to the glycolytic switch, drug-resistant AGS and ACC-201 GC cells showed an increased CAIX expression compared to the relative wild-type/sensitive counterpart, as demonstrated by the membrane-staining of the CAIX (Fig. 4a) and the total CAIX protein content detected in each cell line (Fig. 4b). To further stress the liaison between CAIX and GC drug resistance, we isolated the CAIX high-expressing cells and the CAIX low-expressing cells within the whole AGS wild-type population (CAIX-low = 25% of the WT population; CAIX-high = 25% of the WT population). By comparing these two subpopulations in terms of drug response, we observed that CAIX-high cells expressed a more resistant phenotype than the CAIX-low cells, as showed by the decreased apoptotic levels detected following the treatment with either 5-FU, PTX, CIS, or FLOT (Fig. 4c). Similarly, when forcing the CAIX expression in AGS wild-type cells, CAIX-overexpressing (CAIX+) cells showed a reduced inhibition of proliferation and decreased apoptotic levels compared to the control, following the treatment with either 5-FU, PTX, CIS, or FLOT (Fig. 4d).

3.4. The CAIX inhibitor SLC-0111 increased therapy response in wild-type and sensitized drug-resistant GC cells

The CAIX inhibitor SLC-0111 successfully passed the phase I clinical trial to determine its safety and tolerability in patients with advanced solid tumors [21] and is currently under phase Ib/II clinical trial (NCT03450018) in combination with gemcitabine for metastatic pancreatic ductal cancer in subjects positive for CAIX. We already demonstrated its efficacy in boosting cancer cell response in melanoma, breast, and colorectal carcinoma [20,22]. Here we tested its ability to improve the therapy response of GC cells. We observed that the SLC-0111 alone was able to almost halve the proliferation of both AGS and ACC-201 wild-type cells, and, when used in combination with 5-FU, PTX, CIS or FLOT, it improved drug response in terms of cell proliferation of about 50% in AGS cells and a 30% in ACC-201 cells compared to the single treatments (Fig. 5a, c). Importantly, when administered as a single treatment to drug-resistant GC cells, SLC-0111 was able to reduce the proliferation of about 50% in AGS 5FUr, PTXr, CISr and FLOTr, and boosted the antiproliferative effects of the single chemotherapeutics when used in combinatory treatment (Fig. 5b). In ACC-201 drug-resistant cells, SLC-0111 alone induced a proliferative blockage of about 30% in 5-FUr and PTXr cells, while a greater response of about 50% was obtained in CISr and FLOTr cells. Again, in combinatory treatment, SLC-0111 was able to boost the anti-cancer response of all the chemotherapeutics used (Fig. 5d). Such a proliferative blockage induced by SLC-0111 was accompanied by an impairment of cell viability. Besides showing a slight cytotoxic effect when administered alone in AGS and ACC-201 wild-type cells, SLC-0111 was able to strengthen the cytotoxicity of 5-FU, PTX, CIS, and FLOT treatments in combinatory strategy in both cell lines (Fig. 5e, g). Moreover, when administered to drug-resistant GC cells, a higher level of apoptosis was gained with SLC-0111 used in combination with either 5-FU, PTX, CIS, or FLOT on 5-FUr, PTXr, CISr, and FLOTr AGS and ACC-201 cells compared to the single treatments (Fig. 5f, h). According to these results, colony formation assay confirmed the activity of the SLC-0111 compound in boosting the anti-cancer response induced by the conventional chemotherapeutics in wild-type GC cells and importantly, its ability to sensitize the drug-resistant GC cells back to the treatments (Figs. 6–7). To verify the crucial role of CAIX in sustaining drug resistance, we complemented the observations obtained by using SLC-0111 with CAIX silencing experiments performed on wild-type and FLOTr AGS and ACC-201 cells. We ensured the effectiveness of CAIX silencing by Western blot and flow cytometry analyses to detect the CAIX expression either in the whole cell lysate or on the plasma membrane, respectively. We gained about 70% reduction of the total CAIX expression in wild-type and FLOTr AGS cells, a halved expression in wild-type ACC-201 cells, while the FLOTr ACC-201 cells showed an even further decrease (around 85%) (Supplementary Fig. 1a). Concerning the plasma membrane expression of CAIX, we observed a halved reduction in silenced wild-type AGS and ACC-201 cells while about a 30% decrease was observed in FLOTr AGS and ACC-201 cells (Supplementary Fig. 1b). Then, we subjected wild-type and FLOTr AGS and ACC-201 cells to the FLOT regimen for 72 h and observed that CAIX down-regulation leads to a boosted therapy response in both sensitive and drug-resistant GC cells. In more detail, silenced wild-type AGS suffered a 50% proliferative block compared to the control cells when treated with FLOT, while the FLOTr showed a slightly decreased reduction (around 20%) in proliferation even though still significant compared to the control (Supplementary Fig. 1c, left); similarly, a slight but significant reduction of proliferation following the FLOT treatement was observed in silenced wild-type and FLOTr ACC-201 cells (30% and 25% respectively) compared to the relative control (Supplementary Fig. 1c, right). Accordingly, a significant increase of cleaved caspases 3/7 levels were obtained upon 72 h-FLOT treatment in CAIX-silenced wild-type and FLOTr AGS and ACC-201 cells, indicating a reduced chemoresistance in GC cells when CAIX is down-regulated (Supplementary Fig. 1d). These data support the evidence collected by using the CAIX inhibitor SLC-0111, enforcing the liaison of CAIX and chemoresistance in GC, and pushing the development/usage of clinically available CAIX inhibitors for GC patients.

4. Discussion

CAIX plays a central role in the regulation of the balance between extracellular and intracellular pH by catalyzing the conversion of water and carbon dioxide into carbonic acid. In solid tumors, CAIX activity allows cancer cells to grow and survive in a hostile milieu and is involved in the modulation of several important mechanisms such as adaptation to hypoxic conditions, cell adhesion, migration, invasion, chemoresistance, and eventually metastatic spread [6,7,23–25]. Although CAIX overexpression has been correlated with a poor prognosis in several human cancers [5], its role in GC has not been definitively clarified. This is mainly because gastric healthy mucosa expresses higher levels of CAIX when compared to the corresponding tumor tissue, unlike several other cancer types which instead overexpress CAIX (which is indeed restricted to the healthy gastro-intestinal trait and rather known as a tumor isoform of CAs, together with the CAXII) [26]. Nevertheless, it has been demonstrated that the CAIX tumor/healthy tissue ratio in GC patients increases in parallel with disease progression, and such disbalance correlates with shorter post-operative survival [8].

Here, we showed that CAIX expression correlates with advanced disease and the onset of chemoresistance. In particular, we demonstrated that CAIX was upregulated in GC patients who were resistant to FLOT/FOLFOX perioperative chemotherapeutic regimens. Indeed, a significantly higher score of CAIX expression was observed in patients with limited tumor regression (non-responder group, TRG 3–5) in

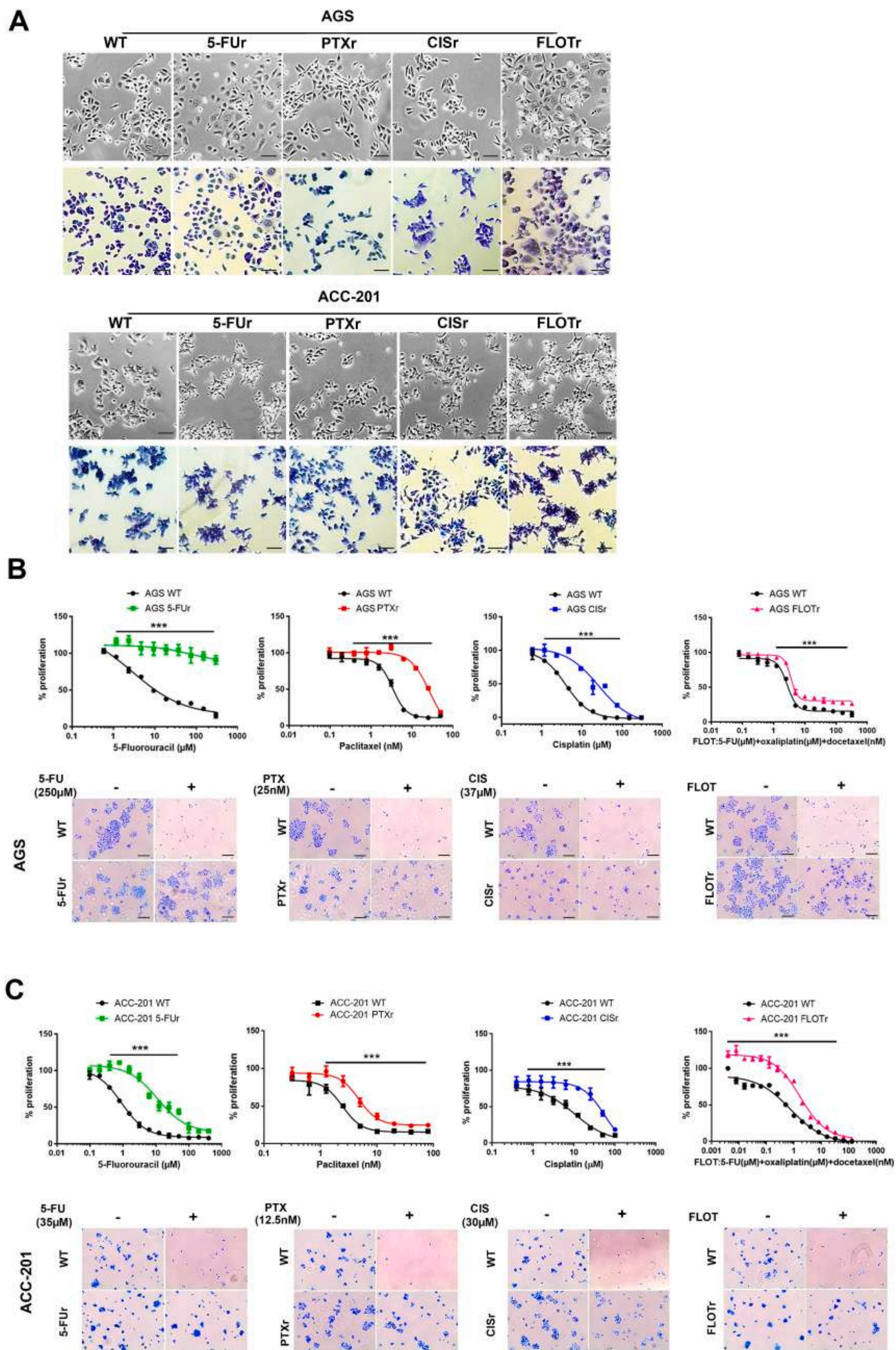


Fig. 2. Drug-resistant GC cells. **a**) Representative pictures and hematoxylin and eosin staining of WT, 5-FUr, PTXr, CISr and FLOTr AGS and ACC-201 cells (scale bar: 250 μm). **b-c**) Growth curves and representative pictures (scale bar: 250 μm) of WT, 5-FUr, PTXr, CISr and FLOTr AGS (**b**) and ACC-201 (**c**) cells following 72 h-treatment with 5-FU, PTX, CIS, or FLOT. Two-Way ANOVA.

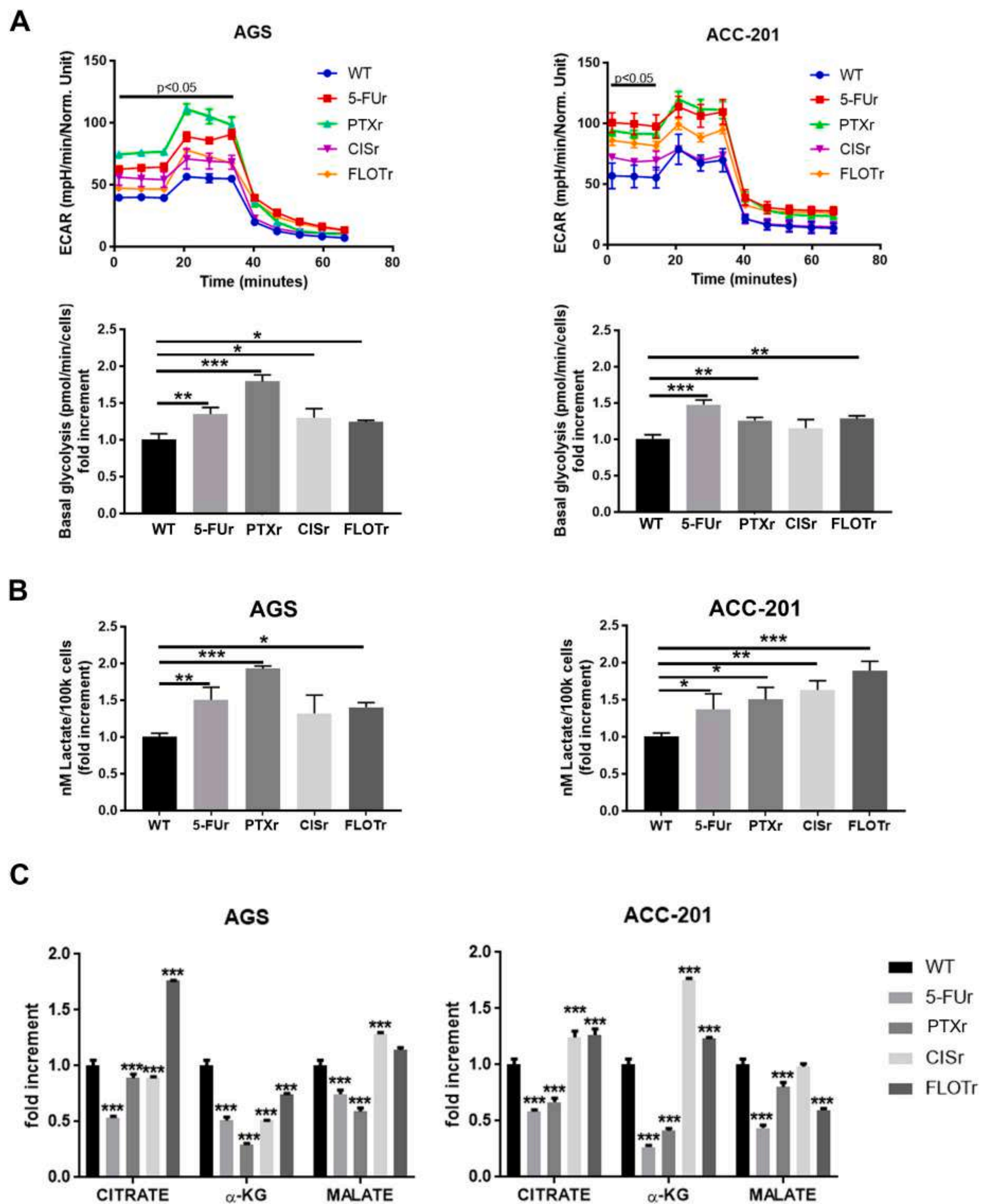


Fig. 3. Metabolic reprogramming of drug-resistant GC cells. Glyco Rate Assay kit performed with the Seahorse XFe96 analyzer (**a**), lactate production (**b**), and GC-MS of the TCA metabolites citrate, α-ketoglutarate and malate (**c**) of WT, 5-FUr, PTXr, CISr, and FLOTr AGS and ACC-201 cells (One-way ANOVA for A and B, Two-Way ANOVA for C; WT *vs* drug-resistant cells).

comparison with patients who experienced a substantial tumor regression (responder group, TRG 2). To validate our observation, we switched to *in vitro* experimentations and generated different chemoresistant GC cell lines, by treating tumor cells with increasing doses of single chemotherapeutic agents typically used for GC treatment, namely 5-FU, CIS, or PTX. Moreover, to deal with a cellular model closer to the clinic scenario where the FLOT regimen is currently used, we also established stable FLOT-resistant GC cell lines by simultaneously combining the 5-FU, Oxaliplatin, and Docetaxel treatments. Notably, we observed that all the chemoresistant cells showed a typical glycolytic phenotype [27–30] accompanied by a higher expression of the CAIX levels compared to their corresponding wild-type. Then, using various procedures we proved that CAIX was significantly associated with drug resistance. Taking into account that some available CAIX inhibitors are currently giving promising results as sensitizers towards conventional chemotherapeutic agents in several pre-clinical settings [9–11], we treated the drug-resistant GC cells with the ureido-benzene-sulphonamide SLC-0111. Importantly, the SLC-0111

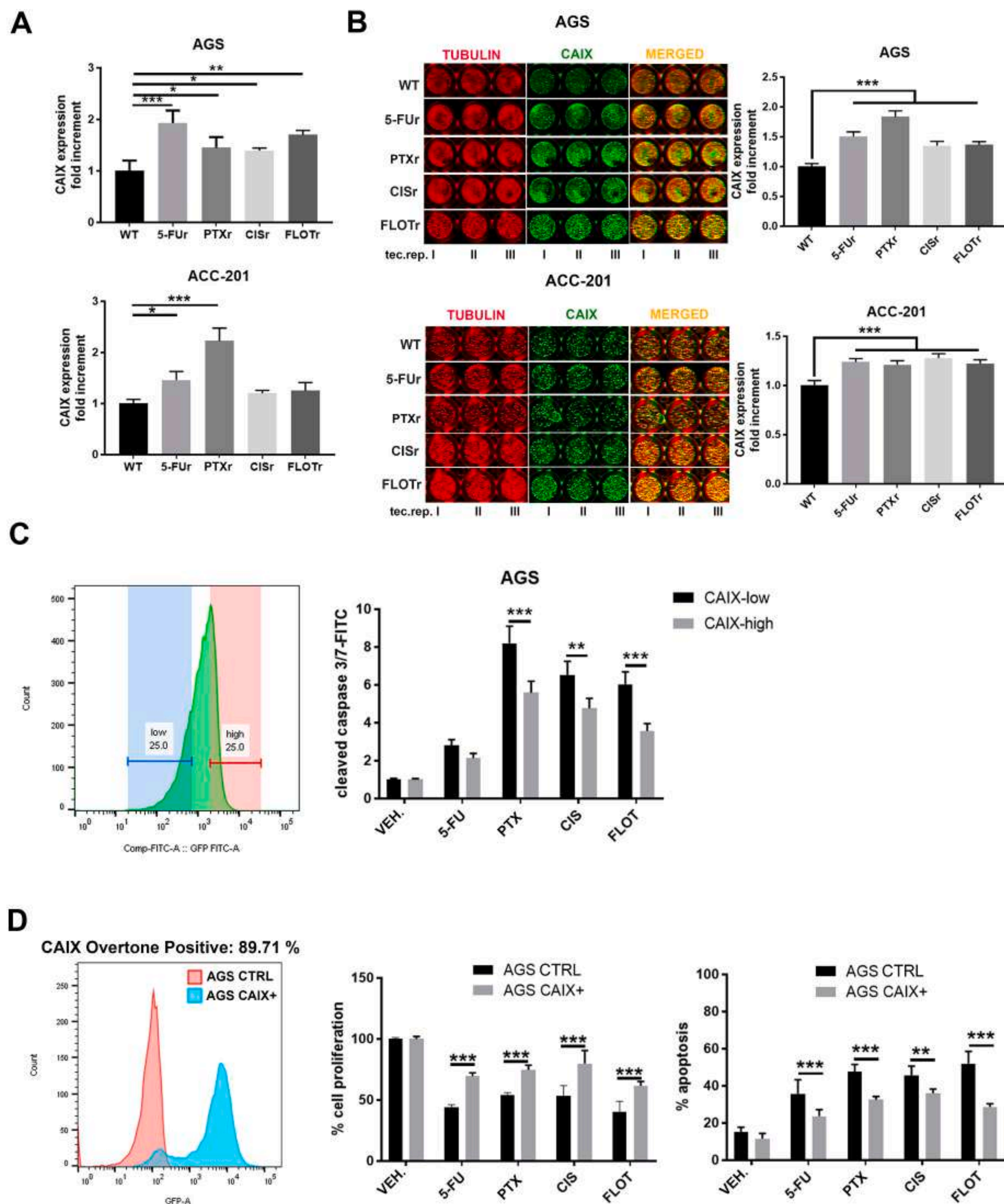


Fig. 4. CAIX expression in wild-type and drug-resistant GC cells. **a-b**) Membrane (**a**) and total (**b**) CAIX expression in WT, 5-FUr, PTXr, CISr, and FLOTr AGS and ACC-201 cells determined by flow cytometry and in-cell western assay, respectively (One-way ANOVA; WT vs drug-resistant cells). **c**) AGS WT sorting of the 25%-high- and the 25%-low-CAIX expressing cells (left). Cleaved caspase3/7 level in CAIX high- and low-expressing AGS cells treated for 72 h with IC50 doses of 5-FU, PTX, CIS, and FLOT (right) (Two-way ANOVA). **d**) Representative flow cytometer plot and overtone value of CAIX expression in control and CAIX + AGS cells (left). MTT assay of CTRL and CAIX + AGS cells treated or not for 72 h with IC50 doses of 5-FU, PTX, CIS, and FLOT (middle) (Two-way ANOVA). Annexin V/PI flow cytometer assay of CTRL and CAIX + AGS cells treated or not for 72 h with IC50 doses of 5-FU, PTX, CIS, and FLOT (right) (Two-way ANOVA).

compound successfully passed the phase I clinical trial evaluation aimed to determine its safety and tolerability in patients with advanced solid tumors [21], and it is currently under phase Ib/II evaluation in combination with gemcitabine for the treatment of metastatic pancreatic cancer. In our *in vitro* models, SLC-0111 administration could significantly impair GC cell proliferation and viability by committing both the wild-type/sensitive and the chemoresistant GC cells to a caspase 3/7-induced apoptosis, and to a reduced colony formation ability. To confirm the importance of CAIX in sustaining chemoresistance, we complemented these observations with CAIX silencing experiments and we obtained similar results showing an increased therapy response in GC cells with down-regulated expression of CAIX than in control cells.

Speculating on how CAIX would contribute to chemoresistance in GC and on the potential mechanisms leading to cancer cells death following CAIX inhibition, we first have to keep in mind that CAIX is a transmembrane enzyme with an extracellular active site able to hydrate CO_2

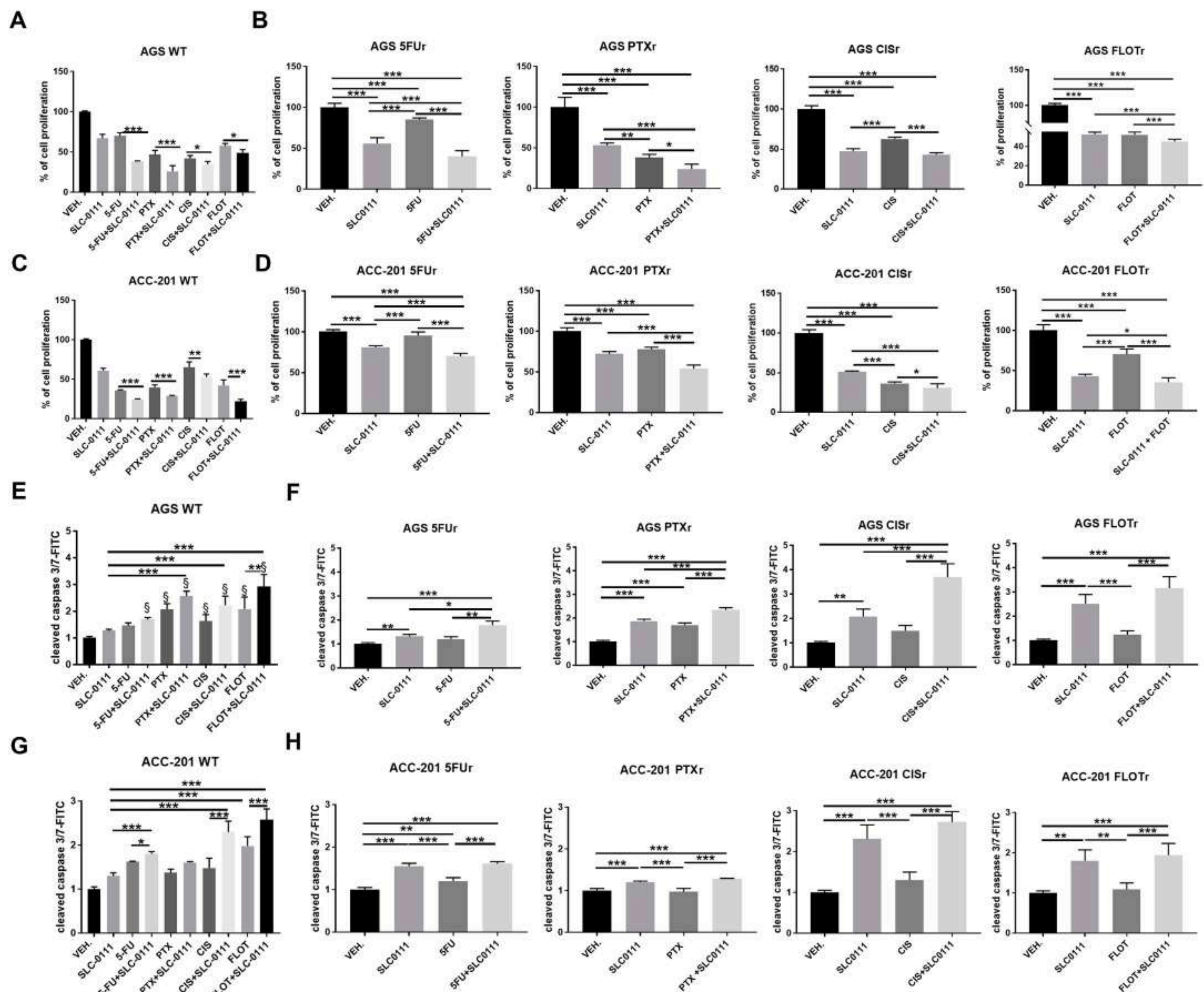


Fig. 5. Cytostatic and cytotoxic effects of SLC-0111 compound on GC cells. a-d) MTT assay of WT, 5-FUr, PTXr, CISr, and FLOTr AGS (**a, b**) and ACC-201 (**c, d**) cells, treated for 72 h with the respective IC50 dose of 5-FU, PTX, CIS, and FLOT as single treatments or in combination with 100 μM SLC-0111 (Two-way ANOVA). **e-h**) Cleaved caspase 3/7 flow cytometer assay performed on WT, 5-FUr, PTXr, CISr, and FLOTr AGS (**e, f**) and ACC-201 (**g, h**) cells, treated for 72 h with the respective IC50 dose of 5-FU, PTX, CIS, and FLOT used as single treatments or in combination with 100 μM SLC-0111 (Two-way ANOVA).

to bicarbonate and protons thereby contributing to the reduction of the extracellular pH (pHe) that is associated with tumor drug resistance [31]. Indeed, inhibitors of CAIX have a great potential to increase the efficiency of chemotherapeutic drugs that are more active at low pHi (internal pH)/pHe such as alkylating agents and platinum-containing compounds. Quite recently, Liu S. and colleagues demonstrated *in vivo* that delivery of acetazolamide-loaded pH-responsive nanoparticles while reducing breast tumor extracellular acidosis enhances the effectiveness of paclitaxel chemotherapy [32]. Further, during inhibition of CAIX enzymatic activity pHi reduces meaning that the internal pH of CAIX-positive tumor cells expresses a significant acidity compromising CAIX-positive tumor cell viability and possibly machinery of drug multi-resistance [33]. Thereby the key role of CAIX to regulate cellular pH makes it an intriguing target to disrupt the pHi/pHe homeostasis and impair the viability of cancer cells.

Based on these findings, we might hypothesize that SLC-0111 administration could help to reverse chemoresistance occurring during GC treatment and thus improve the effect of chemotherapeutic agents without the need to increase their dosage and consequently their side effects.

In conclusion, herein we demonstrated for the first time a positive correlation between CAIX expression and chemoresistance in GC. Moreover, the CAIX inhibitor SLC-0111 seems to improve the anti-proliferative and pro-apoptotic effect of conventional chemotherapeutic agents in both wild-type and chemoresistant GC cells, paving the way for its potential use in a clinical setting in combination with the FLOT/FOLFOX regimens.

Fundings

This work was supported by the University of Florence in Italy and by "Faggi 2020–2022" liberal donation.

Ethical standards

All procedures involving humans were in accordance with the ethical standards of the national responsible committee on human experimentation and with the Helsinki Declaration of 1964 and later versions.

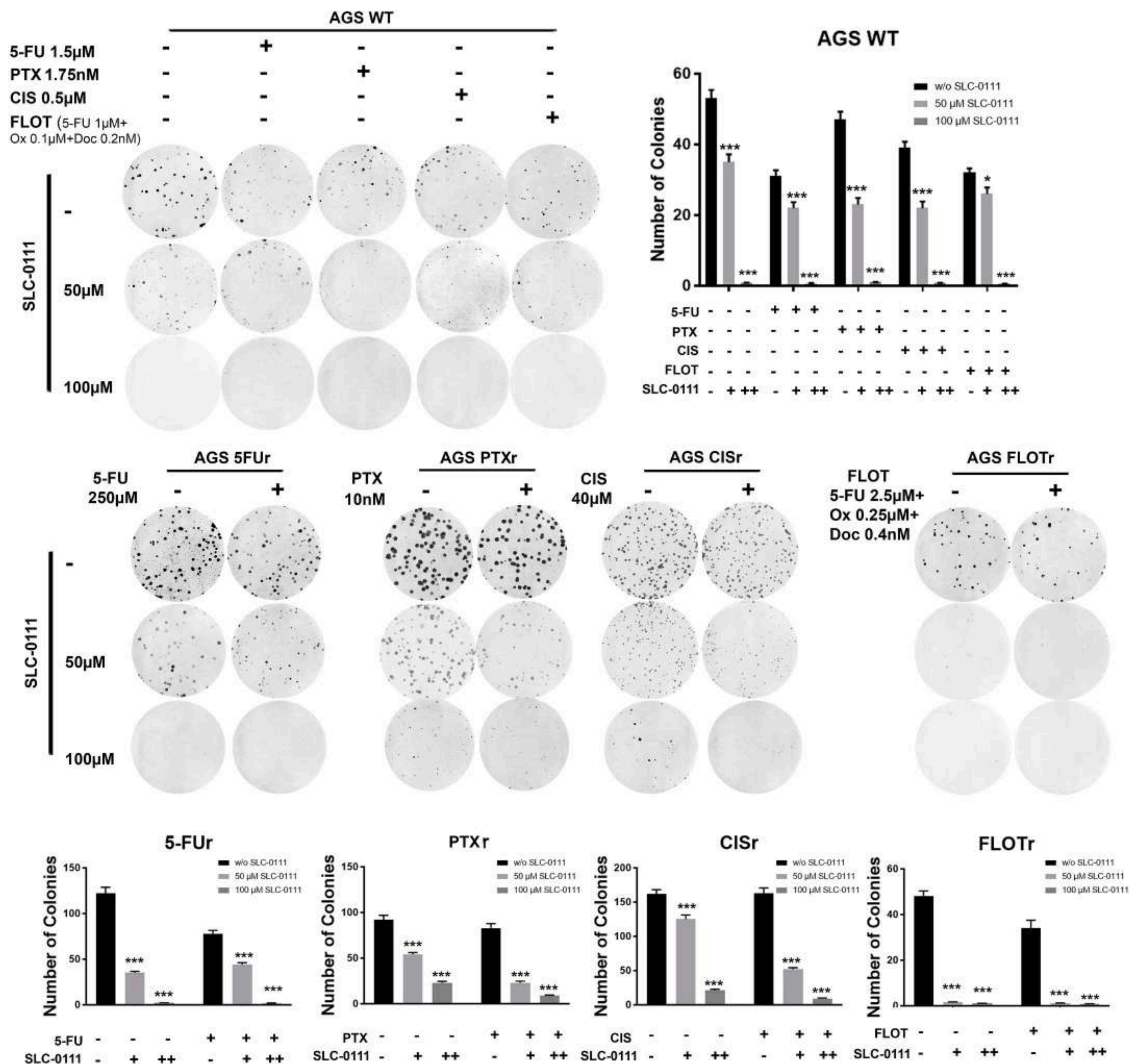


Fig. 6. Colony formation assay of AGS GC cells treated with SLC-0111 alone and in combination with chemotherapy. Colony formation of AGS WT and drug-resistant cells following the treatment with 50 μM (+) and 100 μM (++) SLC-0111 compound as monotherapy and in combination with IC50 doses of 5-FU, PTX, CIS, and FLOT CT. (Two-way ANOVA, vs "w/o SLC-0111" condition).

Informed consent was obtained from all patients. This article does not contain any studies with animal subjects.

CRediT authorship contribution statement

Elena Andreucci: Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Alessio Biagioni:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Sara Peri:** Methodology, Investigation, Formal analysis. **Giampaolo Versienti:** Methodology, Investigation, Formal analysis. **Fabio Cianchi:** Writing – original draft, Resources, Funding acquisition. **Fabio Staderini:** Resources. **Lorenzo Antonuzzo:** Resources. **Claudiu T. Supuran:** Resources. **Erika Olivo:** Investigation. **Elisa Pasqualini:** Investigation. **Luca Messerini:** Investigation. **Daniela Massi:** Resources. **Matteo Lulli:** Validation, Methodology. **Jessica Ruzzolini:** Validation. **Silvia Peppicelli:** Validation. **Francesca Bianchini:** Validation. **Nicola Schiavone:** Funding acquisition. **Lido Calorini:** Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Lucia Magnelli:** Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Laura Papucci:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

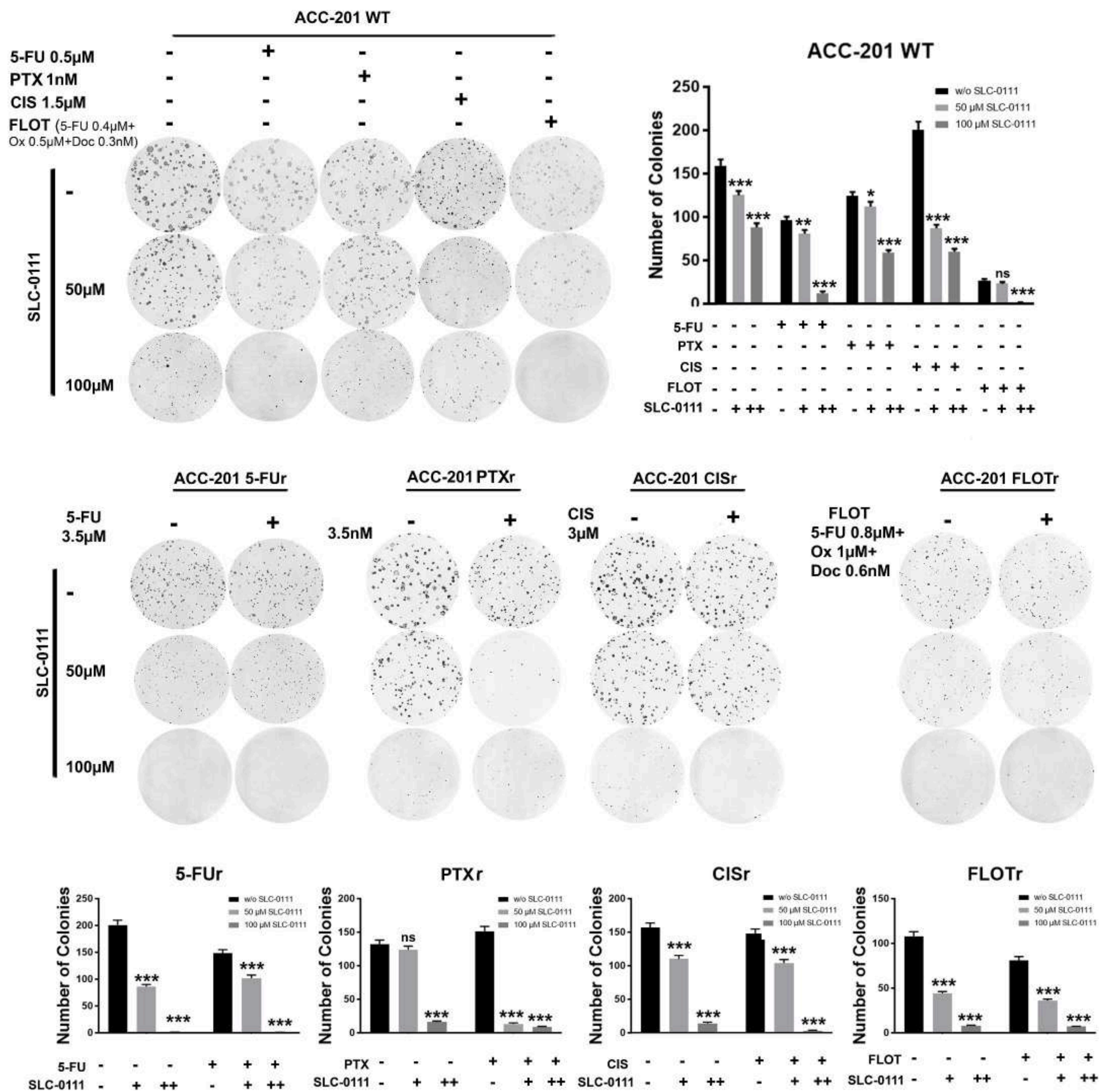


Fig. 7. Colony formation assay of ACC-201 GC cells treated with SLC-0111 alone and in combination with chemotherapy. Colony formation of ACC-201 WT and drug-resistant cells following the treatment with 50 μM (+) and 100 μM (++) SLC-0111 compound as monotherapy and in combination with IC50 doses of 5-FU, PTX, CIS, and FLOT CT. (Two-way ANOVA, vs "w/o SLC-0111" condition).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2023.216338.

References

[1] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, et al., Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA A Cancer J. Clin. 71 (2021) 209–249.

[2] C. Schuhmacher, S. Gretschel, F. Lordick, P. Reichardt, W. Hohenberger, C. F. Eisenberger, et al., Neoadjuvant chemotherapy compared with surgery alone for locally advanced cancer of the stomach and cardia: European Organisation for Research and Treatment of Cancer randomized trial 40954, J. Clin. Oncol. 28 (2010) 5210–5218.

[3] S.-E. Al-Batran, N. Homann, C. Pauligk, T.O. Goetze, J. Meiler, S. Kasper, et al., Perioperative chemotherapy with fluorouracil plus leucovorin, oxaliplatin, and docetaxel versus fluorouracil or capecitabine plus cisplatin and epirubicin for locally advanced, resectable gastric or gastro-oesophageal junction adenocarcinoma (FLOT4): a randomised, phase 2/3 trial, Lancet 393 (2019) 1948–1957.

[4] A. Biagioni, I. Skalamera, S. Peri, N. Schiavone, F. Cianchi, E. Giommoni, et al., Update on gastric cancer treatments and gene therapies, Cancer Metastasis Rev. 38 (2019) 537–548.

[5] A.P. Aldera, D. Govender, Carbonic anhydrase IX: a regulator of pH and participant in carcinogenesis, J. Clin. Pathol. 74 (2021) 350–354.

[6] S. Pastorekova, R.J. Gillies, The role of carbonic anhydrase IX in cancer development: links to hypoxia, acidosis, and beyond, Cancer Metastasis Rev. 38 (2019) 65–77.
[7] D. Neri, C.T. Supuran, Interfering with pH regulation in tumours as a therapeutic strategy, Nat. Rev. Drug Discov. 10 (2011) 767–777.
[8] J. Chen, C. Röcken, J. Hoffmann, S. Krüger, U. Lendeckel, A. Rocco, et al., Expression of carbonic anhydrase 9 at the invasion front of gastric cancers, Gut 54 (2005) 920–927.
[9] E. Andreucci, J. Ruzzolini, S. Peppicelli, F. Bianchini, A. Laurenzana, F. Carta, et al., The carbonic anhydrase IX inhibitor SLC-0111 sensitises cancer cells to conventional chemotherapy, J. Enzym. Inhib. Med. Chem. 34 (2019) 117–123.
[10] S. Peppicelli, E. Andreucci, J. Ruzzolini, F. Bianchini, C. Nediani, C.T. Supuran, et al., The Carbonic Anhydrase IX inhibitor SLC-0111 as emerging agent against the mesenchymal stem cell-derived pro-survival effects on melanoma cells, J. Enzym. Inhib. Med. Chem. 35 (2020) 1185–1193.
[11] C.T. Supuran, Carbonic anhydrase inhibitors: an update on experimental agents for the treatment and imaging of hypoxic tumors, Expet Opin. Invest. Drugs 30 (2021) 1197–1208.
[12] J. Nakamura, Y. Kitajima, K. Kai, K. Hashiguchi, M. Hiraki, H. Noshiro, et al., Expression of hypoxic marker CA IX is regulated by site-specific DNA methylation and is associated with the histology of gastric cancer, Am. J. Pathol. 178 (2011) 515–524.
[13] S. Peri, A. Biagioni, G. Versienti, E. Andreucci, F. Staderini, G. Barbato, et al., Enhanced vasculogenic capacity induced by 5-fluorouracil chemoresistance in a gastric cancer cell line, Int. J. Mol. Sci. 22 (2021) 7698.
[14] H.P. Vollmers, K. Stulle, J. Dämmrich, M. Pfaff, T. Papadopoulos, C. Betz, et al., Characterization of four new gastric cancer cell lines, Virchows Arch. B Cell Pathol. Incl. Mol. Pathol. 63 (1993) 335–343.
[15] F.J. van Kuppeveld, J.T. van der Logt, A.F. Angulo, M.J. van Zoest, W.G. Quint, H. G. Niesters, et al., Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification, Appl. Environ. Microbiol. 58 (1992) 2606–2615.
[16] M. McDermott, A.J. Eustace, S. Busschots, L. Breen, J. Crown, M. Clynes, et al., In vitro development of chemotherapy and targeted therapy drug-resistant cancer cell lines: a practical guide with case studies, Front. Oncol. 4 (2014) 40.
[17] S. Peri, J. Ruzzolini, S. Urciuoli, G. Versienti, A. Biagioni, E. Andreucci, et al., An oleocanthal-enriched EVO oil extract induces the ROS production in gastric cancer cells and potentiates the effect of chemotherapy, Antioxidants 11 (2022) 1762.
[18] E. Andreucci, J. Ruzzolini, F. Bianchini, G. Versienti, A. Biagioni, M. Lulli, et al., miR-214-Enriched extracellular vesicles released by acid-adapted melanoma cells promote inflammatory macrophage-dependent tumor trans-endothelial migration, Cancers 14 (2022) 5090.
[19] E. Andreucci, F. Margheri, S. Peppicelli, F. Bianchini, J. Ruzzolini, A. Laurenzana, et al., Glycolysis-derived acidic microenvironment as a driver of endothelial dysfunction in systemic sclerosis, Rheumatology 60 (2021) 4508–4519.
[20] E. Andreucci, J. Ruzzolini, S. Peppicelli, F. Bianchini, A. Laurenzana, F. Carta, et al., The carbonic anhydrase IX inhibitor SLC-0111 sensitises cancer cells to conventional chemotherapy, J. Enzym. Inhib. Med. Chem. 34 (2019) 117–123.
[21] P.C. McDonald, S. Chia, P.L. Bedard, Q. Chu, M. Lyle, L. Tang, et al., A phase 1 study of SLC-0111, a novel inhibitor of carbonic anhydrase IX, in patients with advanced solid tumors, Am. J. Clin. Oncol. 43 (2020) 484–490.
[22] S. Peppicelli, E. Andreucci, J. Ruzzolini, F. Bianchini, C. Nediani, C.T. Supuran, et al., The Carbonic Anhydrase IX inhibitor SLC-0111 as emerging agent against the mesenchymal stem cell-derived pro-survival effects on melanoma cells, J. Enzym. Inhib. Med. Chem. 35 (2020) 1185–1193.
[23] A. Gibadulinova, P. Bullova, H. Strnad, K. Pohlodek, D. Jurkovicova, M. Takacova, et al., CAIX-mediated control of LIN28/let-7 Axis contributes to metabolic adaptation of breast cancer cells to hypoxia, Int. J. Mol. Sci. 21 (2020) 4299.
[24] S. Daunys, V. Petrikaitė, The roles of carbonic anhydrases IX and XII in cancer cell adhesion, migration, invasion and metastasis, Biol. Cell. 112 (2020) 383–397.
[25] T. Sowa, T. Menju, T.F. Chen-Yoshikawa, K. Takahashi, S. Nishikawa, T. Nakanishi, et al., Hypoxia-inducible factor 1 promotes chemoresistance of lung cancer by inducing carbonic anhydrase IX expression, Cancer Med. 6 (2017) 288–297.
[26] H.M. Becker, Carbonic anhydrase IX and acid transport in cancer, Br. J. Cancer 122 (2020) 157–167.
[27] F. Marcucci, C. Rumio, Glycolysis-induced drug resistance in tumors—a response to danger signals? Neoplasia 23 (2021) 234–245.
[28] T. Wang, K. Ning, X. Sun, C. Zhang, L. Jin, D. Hua, Glycolysis is essential for chemoresistance induced by transient receptor potential channel C5 in colorectal cancer, BMC Cancer 18 (2018) 207.
[29] H. Wu, J. Du, C. Li, H. Li, H. Guo, Z. Li, Kaempferol can reverse the 5-fu resistance of colorectal cancer cells by inhibiting PKM2-mediated glycolysis, IJMS 23 (2022) 3544.
[30] J.-X. Jiang, S. Gao, Y.-Z. Pan, C. Yu, C.-Y. Sun, Overexpression of microRNA-125b sensitizes human hepatocellular carcinoma cells to 5-fluorouracil through inhibition of glycolysis by targeting hexokinase II, Mol. Med. Rep. 10 (2014) 995–1002.
[31] S.-H. Lee, D. McIntyre, D. Honess, A. Hulikova, J. Pacheco-Torres, S. Cerdán, et al., Carbonic anhydrase IX is a pH-stat that sets an acidic tumour extracellular pH in vivo, Br. J. Cancer 119 (2018) 622–630.
[32] S. Liu, X. Luo, S. Liu, P. Xu, J. Wang, Y. Hu, Acetazolamide-loaded pH-responsive nanoparticles alleviating tumor acidosis to enhance chemotherapy effects, Macromol. Biosci. 19 (2019), 1800366.
[33] A. Podolski-Renić, J. Dinić, T. Stanković, M. Jovanović, A. Ramović, A. Pustenko, et al., Sulfocoumarins, specific carbonic anhydrase IX and XII inhibitors, interact with cancer multidrug resistant phenotype through pH regulation and reverse P-glycoprotein mediated resistance, Eur. J. Pharmaceut. Sci. 138 (2019), 105012.