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Cytostatic Bacterial Metabolites Interfere with 5-Fluorouracil and Paclitaxel Efficiency in 4T1 Breast Cancer Cells

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Abstract: The microbiome is capable of modulating the bioavailability of chemotherapy drugs mainly due to metabolizing these agents. Multiple cytostatic bacterial metabolites were recently identified that have cytostatic properties on cancer cells. In this study we addressed the question whether the cytostatic bacterial metabolites (cadaverine, indolepropionic acid, indoxylsulfate) can interfere with the cytostatic effects of the chemotherapy agents used in the management of breast cancer (doxorubicin, gemcitabine, irinotecan, methotrexate, rucaparib, 5-fluorouracil, paclitaxel). The chemotherapy drugs were applied in a wide concentration range to which a bacterial metabolite was added in a concentration within its serum reference range and the effects on cell proliferation were assessed. There was no interference between doxorubicin, gemcitabine, irinotecan, methotrexate, rucaparib and the bacterial metabolites. Nevertheless, cadaverine increased the Hill coefficient of the inhibitory curve of 5-fluorouracil and indolepropionic acid increased the IC_{50} value of 5-fluorouracil that are either disadvantageous effects or effects of unknown significance. Nevertheless, indolepropionic acid decreased the IC_{50} value of paclitaxel that is a potentially advantageous combination.

Keywords: 5-fluorouracil; doxorubicin; gemcitabine; irinotecan; methotrexate; rucaparib; paclitaxel; cadaverine; indolepropionic acid; indoxylsulfate; cell proliferation; breast cancer

1. Introduction

Oncobiosis is the dysbiosis associated with neoplastic diseases. Oncobiosis associates with numerous cancers and affects multiple microbiome compartments [1–3]. There are three major pathways through which the oncobiome can supports tumor progression and metastasis formation: 1) direct colonization of the tumor tissue, 2) immune suppression, 3) the production of bacterial metabolites and toxins [3]. Although, these pathways are all active in breast cancer, metabolite production has key role [3].

Multiple bacterial metabolites were identified with cytostatic [3–14], pro-proliferative [15–24] or mixed [14] properties in breast cancer. These metabolites are chemically very diverse. Metabolites with cytostatic properties elicit pleiotropic effects involving the induction of an anti-Warburg type metabolic rearrangement and the induction of mild oxidative stress that block epithelial-

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mesenchymal transition, reduction of the proportions of cancer stem cells, culminating in cytostasis and reduced metastatic and recurrence potential [3].

There are numerous reports showing that the microbiome interferes with the metabolism and the effectiveness of the chemotherapy agents used in breast cancer management [25–40]. This raised a possibility for other indirect interactions, namely, cytostatic metabolites may add on or potentiate the effectiveness of chemotherapy agents. In this study we set out to investigate that possibility in a cell model of breast cancer.

2. Results

2.1. General Consideratons

For the studies we selected three well-characterized cytostatic bacterial metabolites, cadaverine (CAD), indolepropionic acid (IPA) and indoxylsulfate (IS) that were applied in concentrations corresponding to the top of the serum reference concentration of these metabolites as follows: CAD: 0.8 μM [41,42], IPA: 1 μM [43–45], IS: 4 μM [46]. We investigated doxorubicin (DOX), gemcitabine (GEM), irinotecan (IRI), methotrexate (MTX), rucaparib (RUCA), 5-fluorouracil (5FU) and paclitaxel (PAC) all applied in a serial dilution series as indicated on the corresponding figures (similar to [47] or [48]).

2.2. Bacterial Metabolites Do Not Interfere with Doxorubicin, Gemcitabine, Irinotecan, Methotrexate and Rucaparib Activity

We tested the effects of CAD, IS and IPA on the inhibitory properties of DOX, GEM, IRI, MTX and RUCA on cell proliferation. None of the metabolites impacted on the inhibitory activity of the chemotherapeutic agents, neither on the overall presentation of the inhibitory curves, nor on the kinetic readouts as the IC_{50} value or the Hill coefficient (Figures 1–5).

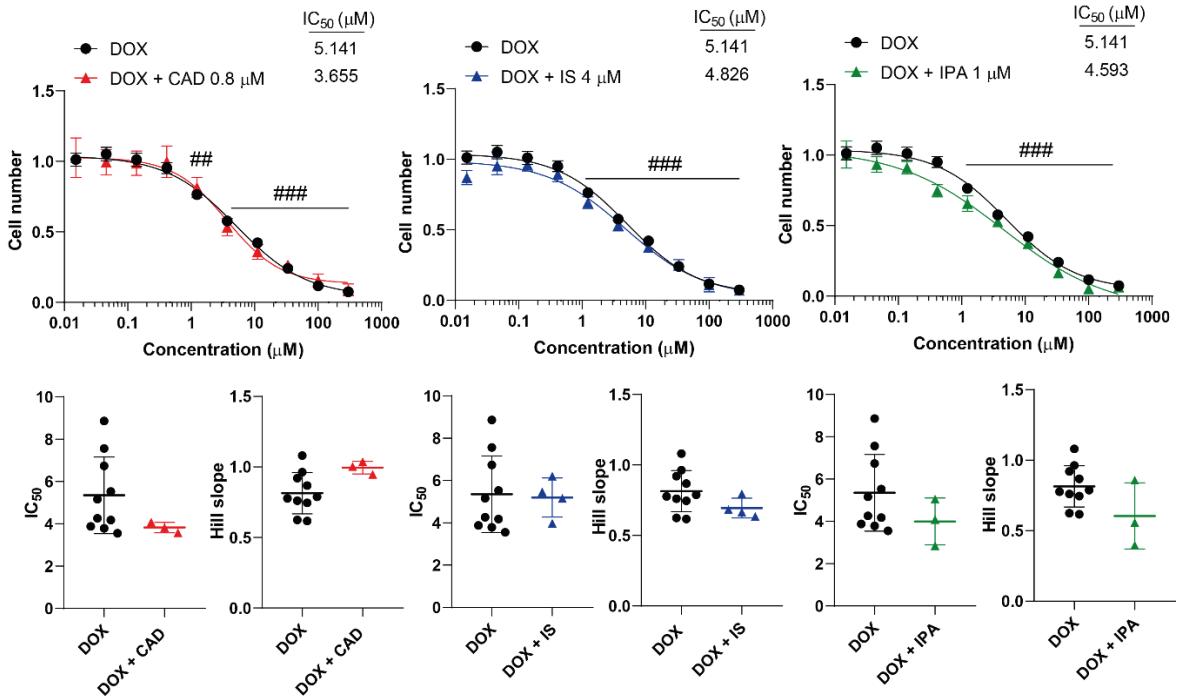

Figure 1. Cytostatic bacterial metabolites do not interfere with the cytostatic effect of doxorubicin. 4T1 cells were plated in 96-well plates (1500 cells/well). Cells were treated with doxorubicin alone or in combination with CAD (0.8 μM), IS (4 μM) or IPA (1 μM) for 48 hours, then cell numbers were determined by MTT assay. Data are presented as means ± SEM, from at least three biological replicates. Individual assays were measured in quadruplicate or in triplicate. Values were normalized to vehicle-treated cells (absorbance is equal to 1). Nonlinear regression (Graphpad "[Inhibitor] vs. response (four parameters)" utility) was performed on datasets to obtain IC_{50} and Hill slope values.

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Normality was determined for the inhibitory curves using the D'Agostino and Pearson normality test, while for the IC_{50} values or the Hill slope values using the Shapiro-Wilk test. Statistical difference between the inhibitory curves was determined using a two-way ANOVA test, and all data points were compared with each other (in Tukey post hoc tests). For the comparison of the IC_{50} and Hill slope values non-paired, two-sided t-test was applied. ## and ### indicate $p < 0.01$ and $p < 0.001$, respectively, DOX-treated vs. vehicle-treated cells. Abbreviations: CAD – cadaverine, DOX–doxorubicin, IPA – indolepropionic acid, IS – indoxylsulfate.

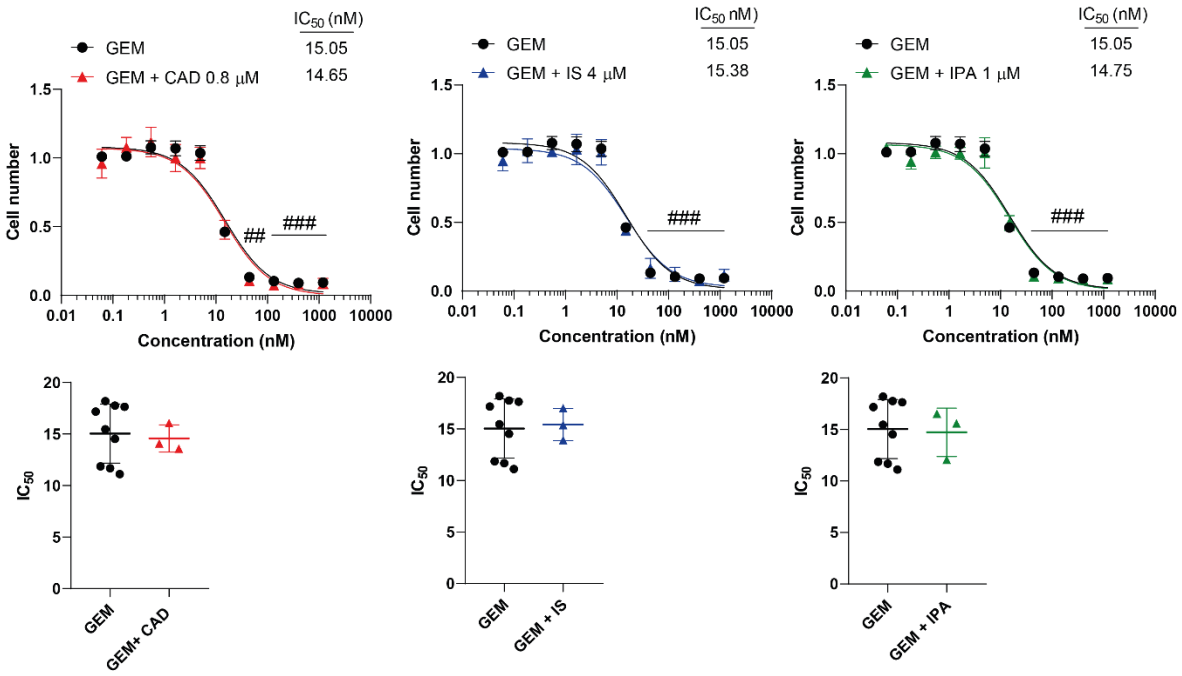

Figure 2. Cytostatic bacterial metabolites do not interfere with the cytostatic effect of gemcitabine. 4T1 cells were plated in 96-well plates (1500 cells/well). Cells were treated with gemcitabine alone or in combination with CAD (0.8 μM), IS (4 μM) or IPA (1 μM) for 48 hours, then cell numbers were determined by MTT assay. Data are presented as means ± SEM, from at least three biological replicates. Individual assays were measured in quadruplicate or in triplicate. Values were normalized to vehicle-treated cells (absorbance is equal to 1). Nonlinear regression (Graphpad "[Inhibitor] vs. response (three parameters)" utility) was performed on datasets to obtain IC_{50} values. Normality was determined for the inhibitory curves using the D'Agostino and Pearson normality test, while for the IC_{50} values using the Shapiro-Wilk test. Dataset normality was achieved by the Box-Cox normalization method. Statistical difference between the inhibitory curves was performed using a two-way ANOVA test, and all data points were compared with each other (in Tukey post hoc tests). For the comparison of the IC_{50} values non-paired, two-sided t-test was applied. ## and ### indicate $p < 0.01$ and $p < 0.001$, respectively, GEM-treated vs. vehicle-treated cells. Abbreviations: CAD – cadaverine, GEM – gemcitabine, IPA – indolepropionic acid, IS – indoxylsulfate.

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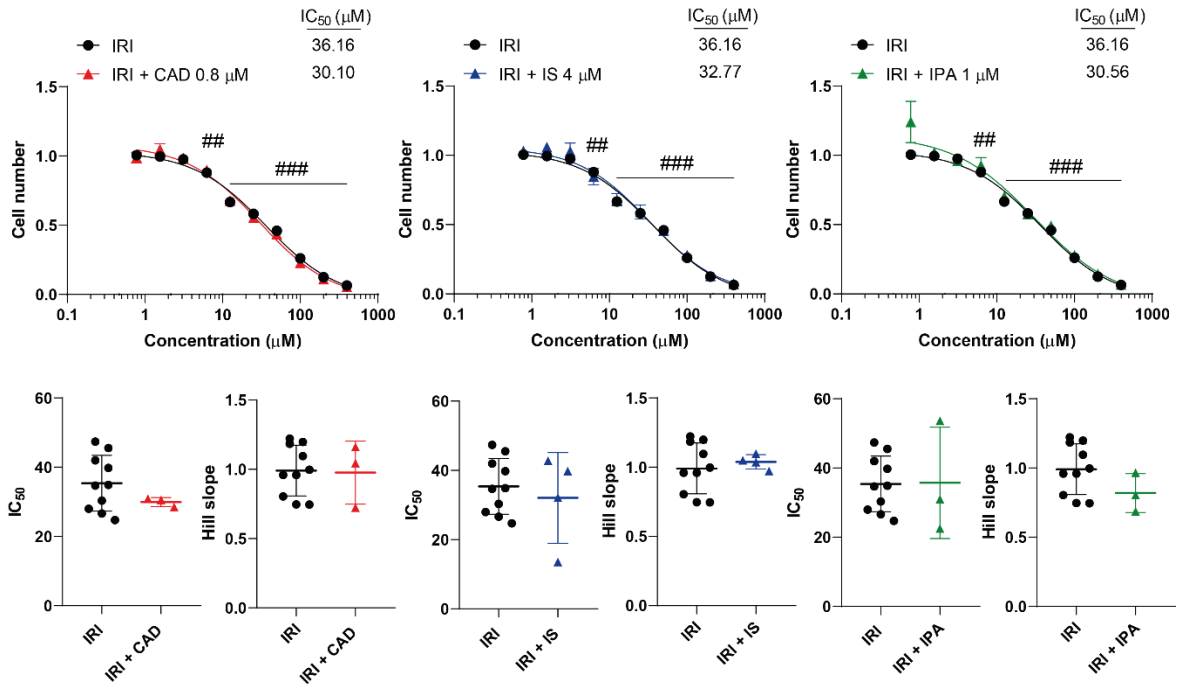

Figure 3. Cytostatic bacterial metabolites do not interfere with the cytostatic effect of irinotecan. 4T1 cells were plated in 96-well plates (1500 cells/well). Cells were treated with irinotecan alone or in combination with CAD (0.8 μM), IS (4 μM) or IPA (1 μM) for 48 hours, then cell numbers were determined by MTT assay. Data are presented as means ± SEM, from at least three biological replicates. Individual assays were measured in quadruplicate or in triplicate. Values were normalized to vehicle-treated cells (absorbance is equal to 1). Nonlinear regression (Graphpad "[Inhibitor] vs. response (four parameters)" utility) was performed on datasets to obtain IC_{50} and Hill slope values. Normality was determined for the inhibitory curves using the D'Agostino and Pearson normality test, while for the IC_{50} values or the Hill slope values using the Shapiro-Wilk test. Statistical difference between the inhibitory curves was performed using a two-way ANOVA test, and all data points were compared with each other (in Tukey post hoc tests). For the comparison of the IC_{50} and Hill slope values non-paired, two-sided t-test was applied. ## and ### indicate $p < 0.01$ and $p < 0.001$, respectively, IRI-treated vs. vehicle-treated cells. Abbreviations: CAD – cadaverine, IPA – indolepropionic acid, IRI – irinotecan, IS – indoxylsulfate.

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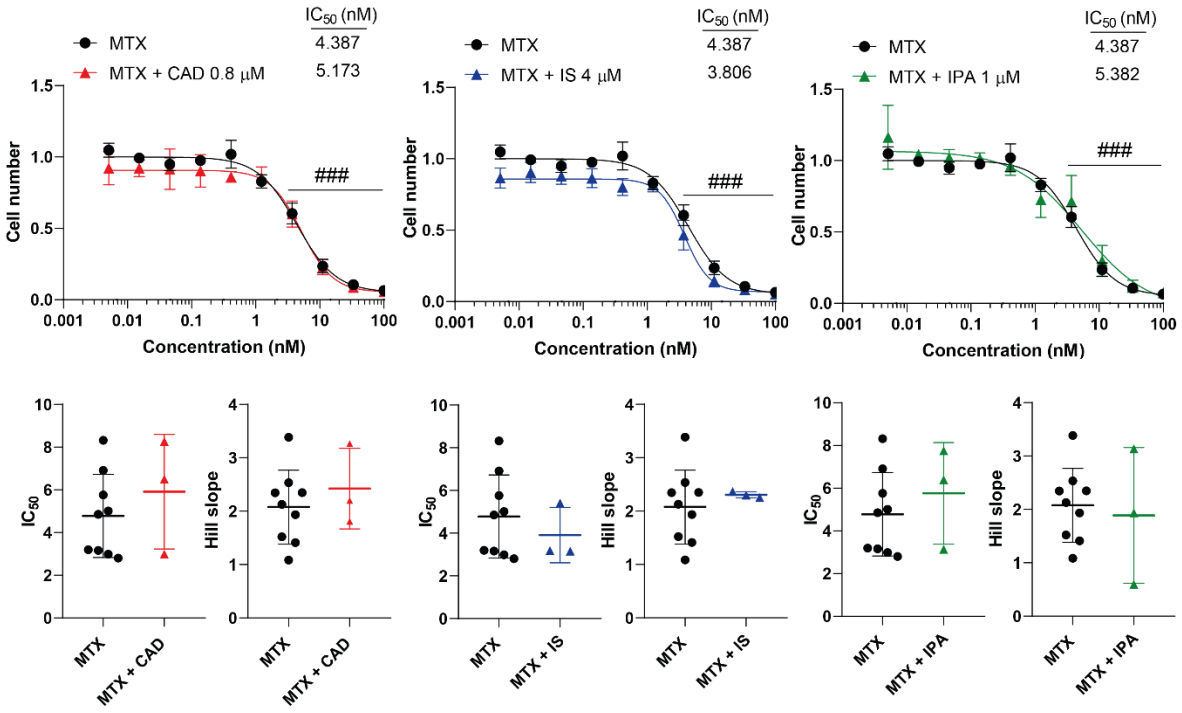

Figure 4. Cytostatic bacterial metabolites do not interfere with the cytostatic effect of methotrexate. 4T1 cells were plated in 96-well plates (1500 cells/well). Cells were treated with methotrexate alone or in combination with CAD (0.8 μM), IS (4 μM) or IPA (1 μM) for 48 hours, then cell numbers were determined by MTT assay. Data are presented as means ± SEM, from at least three biological replicates. Individual assays were measured in quadruplicate or in triplicate. Values were normalized to vehicle-treated cells (absorbance is equal to 1). Nonlinear regression (Graphpad "[Inhibitor] vs. response (four parameters)" utility) was performed on datasets to obtain IC_{50} and Hill slope values. Normality was determined for the inhibitory curves using the D'Agostino and Pearson normality test, while for the IC_{50} values or the Hill slope values using the Shapiro-Wilk test. Statistical difference between the inhibitory curves was performed using a two-way ANOVA test, and all data points were compared with each other (in Tukey post hoc tests). For the comparison of the IC_{50} and Hill slope values non-paired, two-sided t-test was applied. ### indicate $p < 0.001$, respectively, MTX-treated vs. vehicle-treated cells. Abbreviations: CAD – cadaverine, IPA – indolepropionic acid, IS – indoxylsulfate, MTX – methotrexate.

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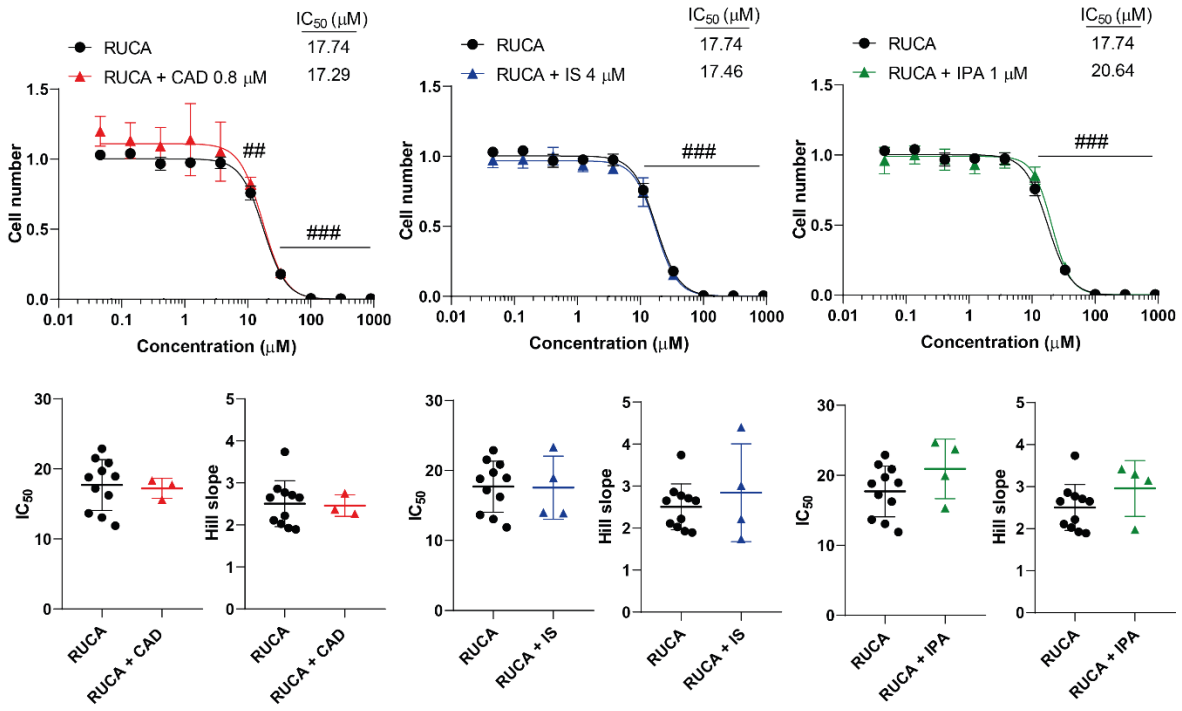

Figure 5. Cytostatic bacterial metabolites do not interfere with the cytostatic effect of rucaparib. 4T1 cells were plated in 96-well plates (1500 cells/well). Cells were treated with rucaparib alone or in combination with CAD (0.8 μM), IS (4 μM) or IPA (1 μM) for 48 hours, then cell numbers were determined by MTT assay. Data are presented as means ± SEM, from at least three biological replicates. Individual assays were measured in quadruplicate or in triplicate. Values were normalized to vehicle-treated cells (absorbance is equal to 1). Nonlinear regression (Graphpad "[Inhibitor] vs. response (four parameters)" utility) was performed on datasets to obtain IC_{50} and Hill slope values. Normality was determined for the inhibitory curves using the D'Agostino and Pearson normality test, while for the IC_{50} values or the Hill slope values using the Shapiro-Wilk test. Statistical difference between the inhibitory curves was performed using a two-way ANOVA test, and all data points were compared with each other (in Tukey post hoc tests). For the comparison of the IC_{50} and Hill slope values non-paired, two-sided t-test was applied. ## and ### indicate $p < 0.01$ and $p < 0.001$, respectively, RUCA-treated vs. vehicle-treated cells. Abbreviations: CAD – cadaverine, IPA – indolepropionic acid, IS – indoxylsulfate, RUCA – rucaparib.

2.3. Bacterial Metabolites Interfere with 5-Fluorouracil

The three bacterial metabolites were tested together with 5FU, an antimetabolite chemotherapeutic agent. CAD increased the Hill coefficient, but did not change the IC_{50} value (Figure 6). Unfortunately, IPA increased the IC_{50} value of 5FU but did not affect the Hill coefficient (Figure 6). IS did not impact on the kinetic values of 5FU (Figure 6).

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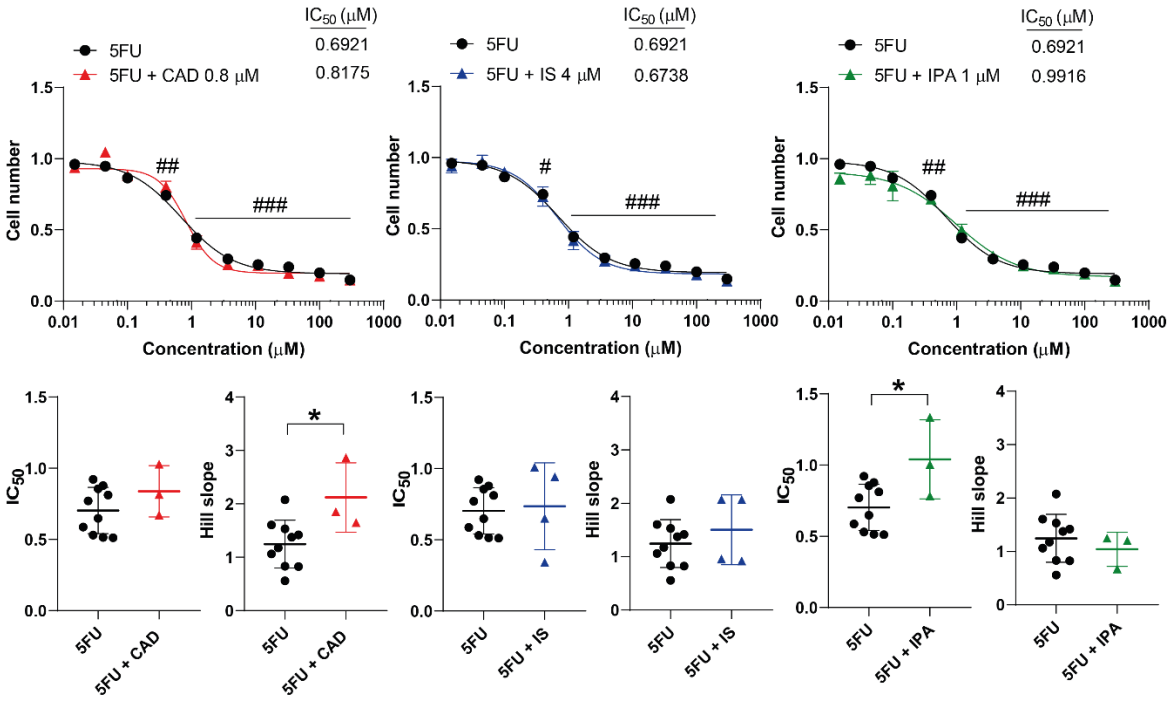

Figure 6. Cadaverine and indolepropionic acid interfere with the cytostatic effects of 5-fluorouracil. 4T1 cells were plated in 96-well plates (1500 cells/well). Cells were treated with 5-fluorouracil alone or in combination with CAD (0.8 μM), IS (4 μM) or IPA (1 μM) for 48 hours, then cell numbers were determined by MTT assay. Data are presented as means ± SEM, from at least three biological replicates. Individual assays were measured in quadruplicate or in triplicate. Values were normalized to vehicle-treated cells (absorbance is equal to 1). Nonlinear regression (Graphpad "[Inhibitor] vs. response (four parameters)" utility) was performed on datasets to obtain IC_{50} and Hill slope values. Normality was determined for the inhibitory curves using the D'Agostino and Pearson normality test, while for the IC_{50} values or the Hill slope values using the Shapiro-Wilk test. To achieve normal distribution, datasets were log-normalized. Statistical difference between the inhibitory curves was performed using a two-way ANOVA test, and all data points were compared with each other (in Tukey post hoc tests). For the comparison of the IC_{50} and Hill slope values non-paired, two-sided t-test was applied. #, ##, and ### indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, 5FU-treated vs. vehicle-treated cells. * represents significance at $p < 0.05$ between the indicated groups. Abbreviations: CAD – cadaverine, IPA – indolepropionic acid, IS – indoxylsulfate, 5FU – 5-fluorouracil.

2.4. Bacterial Metabolites Interfere with Paclitaxel

PAC is an antimicrotubule agent, it interferes with microtubules during cell division. IPA decreased the IC_{50} value of PAC, while leaving Hill coefficient unmodified (Figure 7). Furthermore, there was no interference with the kinetic values of CAD and IS (Figure 7).

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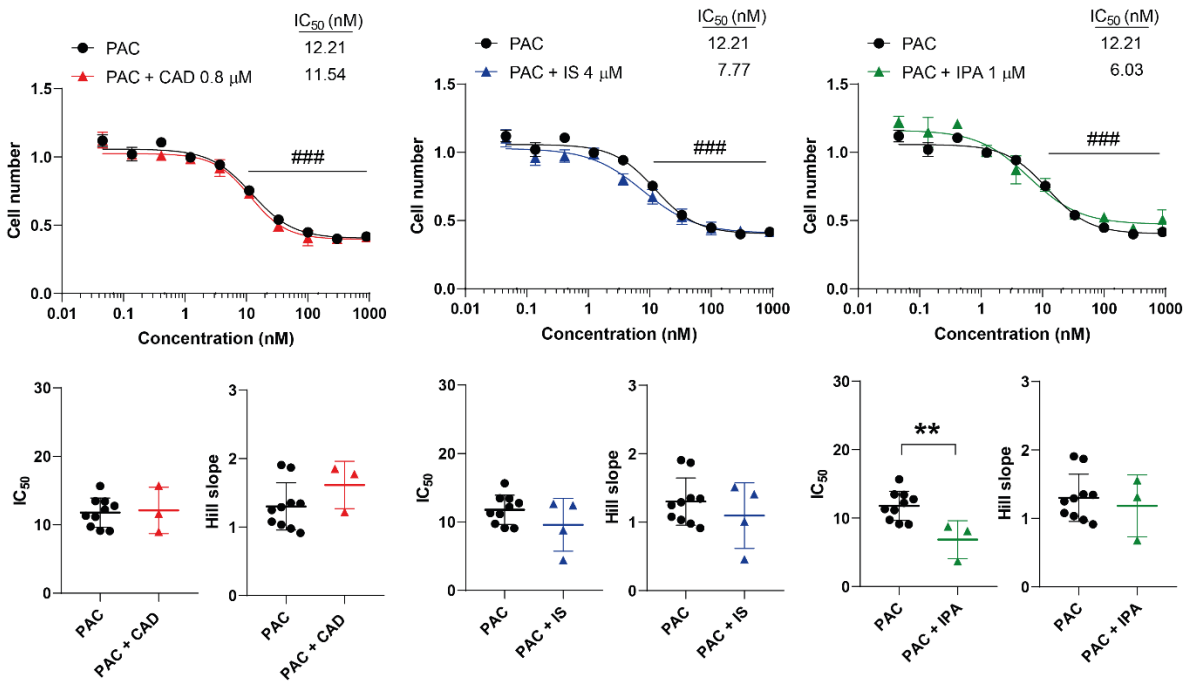

Figure 7. Indolpropionic acid improves the cytostatic effect of paclitaxel. 4T1 cells were plated in 96-well plates (1500 cells/well). Cells were treated with paclitaxel alone or in combination with CAD (0.8 μM), IS (4 μM) or IPA (1 μM) for 48 hours, then cell numbers were determined by MTT assay. Data are presented as means ± SEM, from at least three biological replicates. Individual assays were measured in quadruplicate or in triplicate. Values were normalized to vehicle-treated cells (absorbance is equal to 1). Nonlinear regression (Graphpad "[Inhibitor] vs. response (four parameters)" utility) was performed on datasets to obtain IC_{50} and Hill slope values. Normality was determined for the inhibitory curves using the D'Agostino and Pearson normality test, while for the IC_{50} values or the Hill slope values using the Shapiro-Wilk test. Statistical difference between the inhibitory curves was performed using a two-way ANOVA test, and all data points were compared with each other (in Tukey post hoc tests). For the comparison of the IC_{50} and Hill slope values non-paired, two-sided t-test was applied. ### indicate $p < 0.001$, respectively, 5FU-treated vs. non-treated cells. ** represents significance at $p < 0.01$ between the indicated groups. Abbreviations: CAD – cadaverine, IPA – indolepropionic acid, IS – indoxylsulfate, PAC - paclitaxel.

3. Discussion

Chemotherapy plays a pivotal role in the management of breast cancer. The chemotherapy regimens are built on anthracyclines, cyclophosphamides, taxanes, antimetabolites (5-fluorouracil, gemcitabine, capecitabine), navelbine [49], targeted therapeutic agents as trastuzumab, pertuzumab and trastuzumab-emtansine, lapatinib [50], endocrine therapy, including selective estrogen receptor modulators (SERMs), aromatase inhibitors and GNRH-analogs [50] and novel therapeutic agents as PARP inhibitors [51–53] or CDK4/6 (cyclin-dependent kinases) inhibitors [54]. In this study we assessed those inhibitors that can be utilized in cell-based model systems, as their action does not require the activation in the liver, interaction with the immune system (as for humanized antibodies) or systemic endocrine loops (e.g., SERMs).

In this study we investigated whether there is an interaction between cytostatic bacterial metabolites and the above-mentioned chemotherapy agents. Numerous bacterial metabolites were identified, the majority of which have cytostatic properties [3–14]. The production of these metabolites decline in breast cancer patients, nevertheless, administration of minute quantities of these metabolites reduce the mitotic rate and the metastatic potential of the primary tumor [4–7].

Multiple chemotherapy agents were shown to modulate the composition of the microbiome [55,56], bacterial metabolism of chemotherapy agents were also evidenced [32,34–40] and the efficiency of humanized antibodies [57] were also linked to compositional changes to the microbiome.

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These observations raised the possibility that bacterial metabolites may interfere with the cytostatic or cytotoxic effects of chemotherapy agents. Despite our negative observations on the interactions between bile acids and chemotherapy agents in pancreatic adenocarcinoma cells [58,59], in the current study we identified CAD and IPA that do interfere with PAC and 5FU. Neither metabolites displayed toxicity towards non-transformed cells in previous studies and can be applied in low concentrations [5,6].

CAD increased the Hill coefficient of 5FU suggesting a more collaborative binding of the drug molecules [60] that has unknown pharmacological relevance. In contrast, IPA increased the IC_{50} value of 5FU suggesting a lower efficiency that has negative pharmacological and, likely, clinical consequences. While in the case of PAC the IC_{50} value was halved in the presence of IPA making this combination a potential. These findings also suggest that IPA may have adverse effects when PAC+5FU combinations are applied.

IPA is a bacterial metabolite that is the synthesized from tryptophan through deamination by tryptophanase (TnaA) [5,45]. A significant portion of tryptophan (4-6%) undergoes bacterial catabolism [61]. Multiple studies have shown that disturbances to indole/tryptophan metabolism correlates with survival in breast cancer ([6,7,62,63], reviewed in [6]). Our observations extend these by adding that higher IPA levels may support PAC responsiveness.

4. Materials and Methods

Chemicals

Bacterial metabolites (Cadaverine-CAD, cat # C8561; Indoxylsulfate-IS, cat # 13875; Indolepropionic acid-IPA, cat # 220027) were purchased from Sigma-Aldrich (St. Louis, MI, USA). All metabolites were dissolved in dimethyl-sulfoxide (DMSO) at a stock concentration of 100 mM. CAD was used at concentrations of 0.8 μM, IS at 4 μM and IPA at 1 μM, corresponding to normal human serum concentrations of these metabolites [41–46].

Chemotherapy drugs, Irinotecan (IRI, cat # I1406), 5-fluorouracil (5FU, cat # F6627), Methotrexate (MTX, cat # PHR1396), Rucaparib (RUCA, cat # PZ0036) and Gemcitabine (GEM, cat # G6423) were from Sigma-Aldrich. The drugs IRI, 5-FU, MTX and RUCA were dissolved in DMSO at a stock concentration of 100 mM; GEM was dissolved in water at a stock concentration of 100 mM. Liposomal Encapsuled Doxorubicin (DOX-NP, cat # 300112) was purchased from Avanti and a stock solution of 50 mM was prepared. Paclitaxel (PAC, cat # A0451335) was from Thermo Fisher Scientific (Waltham, MA, USA) and 50 mM stock solution was prepared in DMSO.

Chemotherapy compounds were used at different concentrations as indicated in the figures.

Cell Line

The 4T1 breast cancer cell line was obtained from the American Type Culture Collection. Cells were cultured in RPMI-1640 medium (Sigma-Aldrich, cat # R5886) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM L-glutamine and 1% pyruvate at 37°C in a humidified incubator with 5% CO_2. Cells were regularly checked for Mycoplasma contamination.

MTT Assay

4T1 cells were plated in 96-well plates (1.5 x 10^3 cell/well). On the next day cells were treated with chemotherapy agents alone or in combination with bacterial metabolites for 48 h. After treatments cell numbers was determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, cells were treated with MTT solution (0.5 mg/ml) and incubated at 37°C for 90 minutes. Then, culture medium was discarded and the formazan crystals were dissolved in DMSO. The absorbance was measured on a plate reader (Thermo Labsystems Multiskan MS, Walthman, MA, USA) at 540 nm. In the calculations, the absorbance values for the vehicle-treated cells were considered 1, and all treatment were expressed relative to 1.

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Statistical Analysis

Each analyses were performed using GraphPad Prism 8 software. Experiments were repeated at least three times and results are presented as mean ± SEM values. Normal distribution of the values was tested using D'Agostino & Pearson normality test. Where appropriate, values were log-normalized or normalized using Box-Cox normalization method [64]. Nonlinear regression was performed using the GraphPad "[Inhibitor] vs. response - Variable slope (four parameters)" utility, from which IC_{50} and Hill slope values were obtained unless otherwise stated. Two-way analysis of variance test followed by Tukey's honestly significant post hoc test were used for multiple comparisons.

5. Conclusions

The oncobiome was shown to modulate the efficacy or even limit the availability of chemotherapy agents. In this study we showed that, in contrast to previous negative findings in pancreatic adenocarcinoma models, IPA and CAD modulated the cytostatic activity of 5FU and PAC. Importantly, IPA decreased the IC_{50} values of PAC that is a beneficial interaction, as PAC concentrations can be decreased in combination with a low concentration non-toxic compound that may limit the side effects of PAC.

Supplementary Materials: No supplementary material is associated with the manuscript.

Author Contributions: Conceptualization, E.M. and P.B.; methodology, E.M., P.B., E.J. and S.S; software, S.S., P.N and T.I.B.; validation, E.M. and P.B.; formal analysis, E.M., P.B. and S.S.; investigation, S.S., T.I.B. P.N. and E.J..; resources, E.M. and P.B.; data curation, S.S.; writing—original draft preparation, S.S., E.M. and P.B; writing—review and editing, E.M and P.B.; visualization, E.M.; supervision, E.M. and P.B.; project administration, E.M and S.S.; funding acquisition, E.M and P.B. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Primary data of the present manuscript can be found at https://figshare.com/s/6ecb8c6bd8b87284ae3c (DOI: 10.6084/m9.figshare.25678635).

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Conflicts of Interest: Péter Bai is a CEO and shareholder of Holobiont Diagnostics LTD, a developer of cancer diagnostic tests. Other authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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