

# Assessment *in vitro* of interactions between anti-cancer drugs and noncancer drugs commonly used by cancer patients

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Cancer patients often suffer from cancer symptoms, treatment complications and concomitant diseases and are, therefore, often treated with several drugs in addition to anticancer drugs. Whether such drugs, here denoted as ‘concomitant drugs’, have anticancer effects or interact at the tumor cell level with the anticancer drugs is not very well known. The cytotoxic effects of nine concomitant drugs and their interactions with five anti-cancer drugs commonly used for the treatment of colorectal cancer were screened over broad ranges of drug concentrations *in vitro* in the human colon cancer cell line HCT116wt. Seven additional tyrosine kinase inhibitors were included to further evaluate key findings as were primary cultures of tumor cells from patients with colorectal cancer. Cytotoxic effects were evaluated using the fluorometric microculture cytotoxicity assay (FMCA) and interaction analysis was based on Bliss independent interaction analysis. Simvastatin and loperamide, included here as an opioid agonists, were found to have cytotoxic effects on their own at reasonably low concentrations whereas betamethasone, enalapril, ibuprofen, metformin, metoclopramide, metoprolol and paracetamol were inactive also at very high concentrations. Drug interactions

ranged from antagonistic to synergistic over the concentrations tested with a more homogenous pattern of synergy between simvastatin and protein kinase inhibitors in HCT116wt cells. Commonly used concomitant drugs are mostly neither expected to have anticancer effects nor to interact significantly with anticancer drugs frequently used for the treatment of colorectal cancer. However, simvastatin shows both cytotoxic effects on its own and a synergistic interaction profile with protein kinase inhibitors worthwhile to investigate further. *Anti-Cancer Drugs* 34: 92–102 Copyright © 2022 The Author(s). Published by Wolters Kluwer Health, Inc.

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## Background

Cancer patients are mostly elderly and, thus, often have concomitant diseases in addition to cancer-related symptoms and will frequently also suffer from adverse effects induced by anticancer drugs. Therefore, cancer patients frequently are on drugs against concomitant diseases, complications and adverse effects in addition to cancer drugs, for example, drugs against cardiovascular disease, pain and nausea [1–4]. While potential pharmacokinetic and pharmacodynamic interactions at the organism level between such concomitant drugs and between them and co-administrated anticancer drugs have been established [2–4], there is very little research on possible interactions

between anticancer drugs and concomitant drugs on the tumor cell level. Therefore, it was considered relevant to investigate whether commonly used concomitant drugs may interact with anticancer drugs on the tumor cell to either produce synergy or antagonism and also if standard drugs might have anticancer effects on their own. In the present study, these issues were investigated *in vitro* in the colon cancer cell line HCT-116wt as well as in primary cultures of tumor cells from patients with colorectal cancer.

There is a great number of concomitant and anticancer drugs that could be included in a drug interaction study *in vitro*. Based on their common use among cancer patients and to represent various pharmacodynamic principles betamethasone, enalapril, ibuprofen, loperamide, metformin, metoclopramide, metoprolol, paracetamol and simvastatin were selected and denoted as concomitant drugs in the interaction analyses. The major clinical indications for these drugs are indicated in Table 1. For anticancer drugs, the antimetabolite 5-fluorouracil, the epidermal

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**Table 1** Concomitant drugs investigated with their main therapeutic indications and approximate maximal plasma concentrations achievable in patients at recommended doses

Drug	Indications	C <sub>max</sub> (μM)
Betamethasone	Nausea, fatigue	0.76
Enalapril	Hypertension	0.12
Ibuprofen	Pain	245
Loperamide	Diarrhea	0.004
Metformin	Type 2 diabetes	14.4
Metoclopramide	Nausea	0.14
Metoprolol	Hypertension, cardiovascular disease	0.84
Paracetamol	Pain	119
Simvastatin	Hypercholesterolemia, cardiovascular disease	0.51

growth factor receptor (EGFR)-TK inhibitor erlotinib, the multi-kinase inhibitor regorafenib, the topoisomerase I inhibitor irinotecan and the alkylating platinum oxaliplatin were selected because these drugs or mechanistically related drugs are in routine use in the treatment of colorectal cancer. Because simvastatin was found to interact with the tyrosine kinase inhibitor erlotinib, targeting EGFR 1, in the above setting, simvastatin was also tested in an expanded drug panel with additional protein kinase inhibitors in clinical use, that is, gefitinib, imatinib, lapatinib, nintedanib, crizotinib, sorafenib and vemurafenib.

Drug synergy is still a confusing concept and different models have been used to classify interactions as synergistic or antagonistic, for example, Bliss independence and Loewe additivity as well as response surface models [5–7]. However, there is not yet an agreement on a gold standard model for the research on drug interactions. In this work, the interactions were described using the Bliss independence model due to the advantage of a simple and conservative criterion of synergy as well as software support (MacSynergy II [8]) which provides interaction analysis over broad ranges of drug concentrations.

## Material and methods

### Cell preparation, culture and seeding

The human colon cancer cell line HCT116wt, which is encoding Kirsten rat sarcoma virus protein but not encoding B-Raf protein mutated, was obtained from Horizon Discovery Ltd. HCT116wt was cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 μg/ml streptomycin and 60 μg/ml penicillin. The cell line was kept at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> in the incubator. The cells were split twice a week. Cells (2500 cells/well) were seeded to 384-well plates in a 50 μl medium by the pipetting robot BioMek 4000 (Beckman Coulter). One column of wells with medium only served as blank and two columns of wells with cells only served as controls. Four interaction replicates were arranged using the MacSynergy II [8] layout on each 384-well plate.

Tumor samples were obtained from 26 patients with colorectal cancer undergoing cytoreductive surgery and intraperitoneal chemotherapy at the Department of Surgery, Uppsala University Hospital. Tumor sampling

was based on patient informed consent and approved by the Regional Ethical Review Board in Uppsala (Dnr 2007/237). Tumor cells were prepared by collagenase digestion as described [9]. The cells obtained were mostly single cells or small cell clusters with ≥90% viability and with less than 30% contaminating nonmalignant cells, as judged by morphological examinations of May-Grünwald-Giemsa-stained cytocentrifugate preparations. Cell seeding on 384-well plates was identical to that for the cell line.

### Drugs and drug preparation

5-fluorouracil, irinotecan, paracetamol, simvastatin and ibuprofen were purchased from Sigma, whereas metformin, betamethasone, loperamide, enalapril, metoprolol, metoclopramide, oxaliplatin, regorafenib, crizotinib, nintedanib and vemurafenib were purchased from Selleck Chemicals LLC (Texas, USA). Erlotinib, gefitinib, imatinib, lapatinib and sorafenib were purchased from LC Laboratories (Massachusetts, USA).

All drugs were prepared as a high-concentration stock solution, using dimethyl sulfoxide as solvent. Regorafenib was prepared as 10 mM and 0.1 mM stock solutions on the source plate due to limited solubility. The final concentrations for standard drugs ranged from 0.01 to 90 μM. Since the concentration of ibuprofen and paracetamol in patients can go up to 245 and 119 μM, respectively, the highest final concentration of ibuprofen and paracetamol was set to 270 μM in separate control experiments. Data on the highest plasma concentrations (C<sub>max</sub>) achievable of concomitant drugs were obtained from standard clinical pharmacology reviews released by US Food & Drug Administration and are detailed in Table 1. Cytotoxic anticancer drugs final concentrations ranged from 0.25 to 180 μM. The highest concentration for the kinase inhibitors was 90 μM. The drug concentrations selected for the anticancer drugs were selected to cover the full dynamic range of cytotoxic effects in the cytotoxicity analysis.

### Cytotoxicity analysis

Concomitant and anticancer drugs were added to the cell plates by an Echo 550 (Beckman Coulter, California, USA) after the cells had been seeded and preincubated overnight. Anticancer drugs were added approximately 4 h after the addition of concomitant drugs. After drug addition, the cells were incubated for 72 h before assessment of cell viability in the fluorometric microculture cytotoxicity assay (FMCA).

The FMCA is based on the hydrolysis of fluorescein diacetate (FDA) by esterases in cells with intact plasma membranes [10]. After washing the plates with PBS twice, FDA was added into each well by an EL406 washer dispenser (BioTek Instruments Inc, Vermont, USA). The cells were then incubated for 50 min to hydrolyze FDA. The fluorescein signals were then measured by the CLARIOstar microplate reader (BMG Labtech, Germany) [10].

### Data and interaction analysis

Cell survival from the FMCA was reported as survival indices (SI) calculated as:

$$SI \% = \frac{f_{sample} - f_{blank}}{f_{control} - f_{blank}} \times 100,$$

Where  $f_{sample}$  denotes the average fluorescence signal from the well of the experimental wells,  $f_{control}$  denotes the average fluorescence signal from the growth control wells and  $f_{blank}$  denotes the average fluorescence signal from the blank wells.

A Bliss independence model and Bonferroni adjustment for multiple testing were used to evaluate the interactions between concomitant drugs and anticancer drugs [8]. The simplified Bliss independence model evaluates the interaction by the following formula:  $S_p = S_a \times S_b$ , where  $S_p$  denotes the predicted survival of a combination,  $S_a$ ,  $S_b$  respectively denote the observed survival of drug  $a$ ,  $b$  administrated alone. If  $S_o$  denotes the observed survival of a combination, then  $S_p - S_o < 0$  corresponds to a situation where the drug combination kills fewer cells than expected, hence the combination is defined as antagonistic. When  $S_p - S_o > 0$ , the drug combination kills more cells than expected and the combination is defined as synergistic. The interaction between two drugs can be varyingly synergistic and antagonistic at different concentrations. MacSynergy II provides summary measures of antagonism and synergy (corresponding to volumes of the surface  $S_p - S_o$  in regions with significant interactions). Here we summarized the interaction by computing a net interaction score for each drug pair as the difference between the summary measures of synergy and antagonism respectively. Thus, a positive interaction score corresponds to a larger or more pronounced region of synergy than antagonism, negative scores to vice-versa.

### Statistical analysis

Paired Student's  $t$ -test was used for calculations of statistical significance for SI-values in concentration-response curves. One sample  $t$ -test was performed for calculation of statistical significance for the overall interaction (interaction score), calculated as the Bonferroni adjusted difference between total synergy and total antagonism. Statistical calculations as well as graphical presentations were performed in GraphPad Prism, version 7.

### Results

Therapeutic indications for the concomitant drugs investigated in this study as well as their  $C_{max}$  in patients and as reported in standard pharmacy texts are listed in Table 1. Most  $C_{max}$  are well below  $1 \mu\text{M}$ . The  $C_{max}$  for ibuprofen and paracetamol, however, are considerably higher, thus the maximum concentration of these drugs in control experiments was set to  $270 \mu\text{M}$ .

In the HCT116wt monolayer cell line model, most concomitant drugs did not affect cell viability or growth even at high concentrations (Fig. 1). However, simvastatin alone at  $10 \mu\text{M}$  reduced SI to approximately 70% and further down to 30% at  $30 \mu\text{M}$ . Also  $10 \mu\text{M}$  loperamide alone reduced SI to 80% and to 10% at  $30 \mu\text{M}$ . Paracetamol and ibuprofen were essentially without effect even at  $270 \mu\text{M}$  with SIs close to 90% for both drugs (not shown).

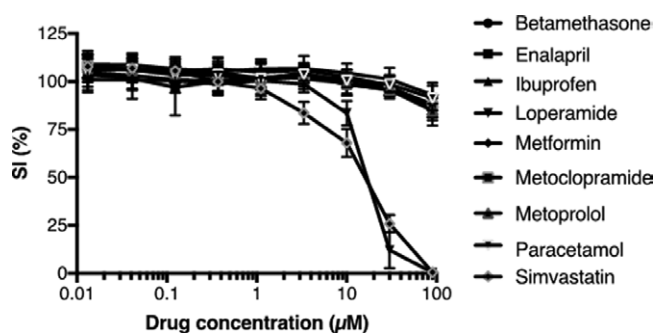
For interactions, 45 combinations of the nine concomitant drugs and the five anticancer drugs over wide concentration ranges were screened. Figure 2 presents the Bonferroni adjusted evaluation of the interactions as reported from the shareware. For each combination, two columns are shown to present the interactions. Column deviations to the right from the 0 line indicate a net amount of synergy whereas columns deviating to the left indicate a net amount of antagonism. Each column represents the sum of the Bonferroni adjusted synergy or antagonism values for the combination over the concentration ranges tested. Loperamide produced a small but statistically significant antagonism when combined with erlotinib whereas simvastatin produced a more obvious and statistically significant synergy with irinotecan and erlotinib. Most other combinations were dominated by minor antagonistic effects.

To more clearly illustrate the interactions observed, the concentration closest to concomitant drug  $C_{max}$  in patients and the concentration that tended to show some minor cytotoxic effect *in vitro*, to indicate a biologic effect in the HCT116 model, were selected for data presentation. Figures 3 and 4 show the results of the concentration-response curves for the five anticancer drugs alone and when combined with loperamide and simvastatin, respectively, the concomitant drugs that produced statistically significant interactions as shown in Fig. 1. Loperamide at  $10 \mu\text{M}$  shifted the concentration-response curve to the left, indicating synergy, when combined with regorafenib, although this was not statistically significant (Fig. 3). The concentration-response curve for loperamide at  $10 \mu\text{M}$  combined with anticancer drugs was generally below that for the anticancer drug alone but this is probably explained by the small cytotoxic effect of loperamide alone. Simvastatin at  $3.3 \mu\text{M}$  clearly and statistically significantly down- and left-shifted the concentration-response curve for erlotinib (Fig. 4).

Interactions for all five anticancer drugs and the nine concomitant drugs presented in this way are shown in Supplementary Figure. S1-S5, Supplemental digital content 1, <http://links.lww.com/ACD/A442>. In separate experiments, we observed that neither ibuprofen nor paracetamol at  $270 \mu\text{M}$  showed any interaction with the anticancer drugs (not shown).

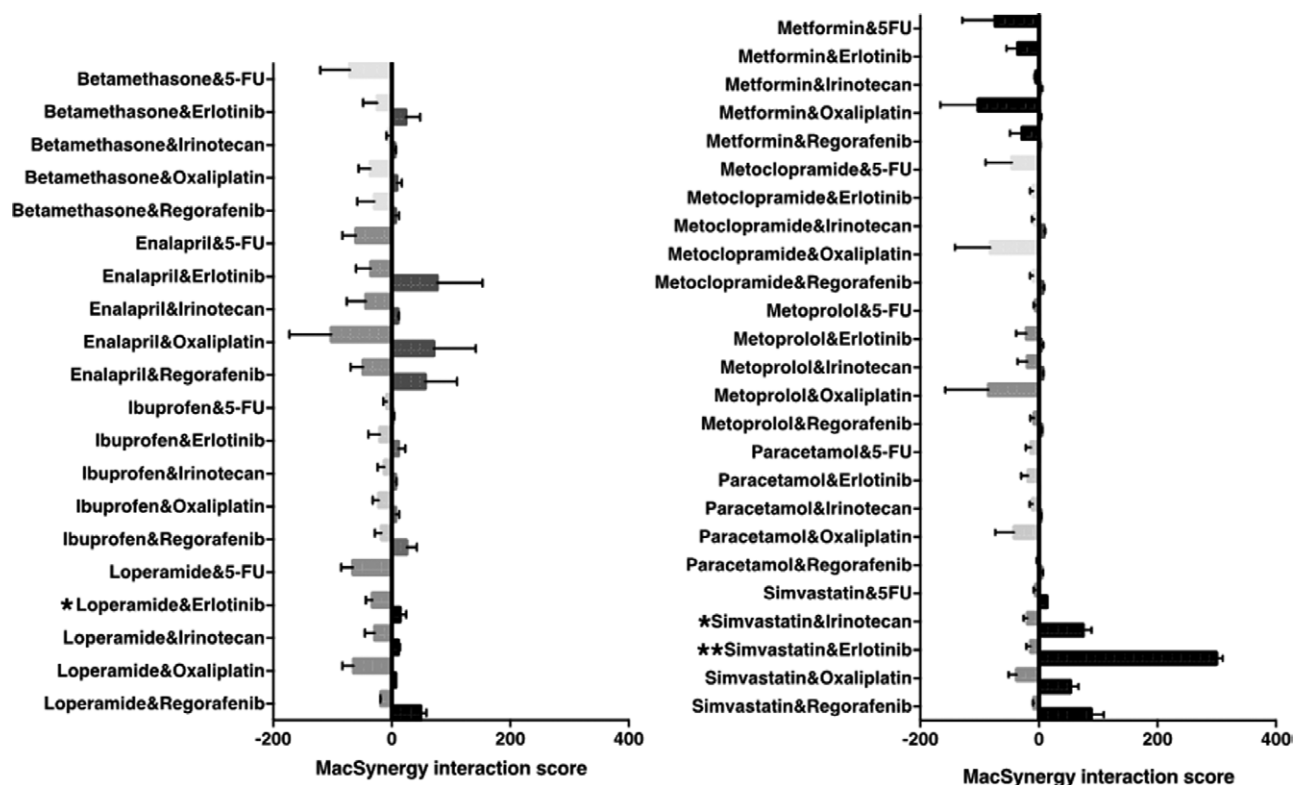
Given the synergy observed between simvastatin and erlotinib (Fig 4, top right) it was of interest to investigate

Fig. 1



Effect of the indicated concomitant drugs alone over a broad range of concentrations on HCT116 wt colon cancer cells. Cell survival was assessed after 72 h of continuous drug exposure and is expressed as survival index (SI) in per cent of unexposed control wells. Results are presented as means  $\pm$  SD of 15 independent experiments.

Fig. 2



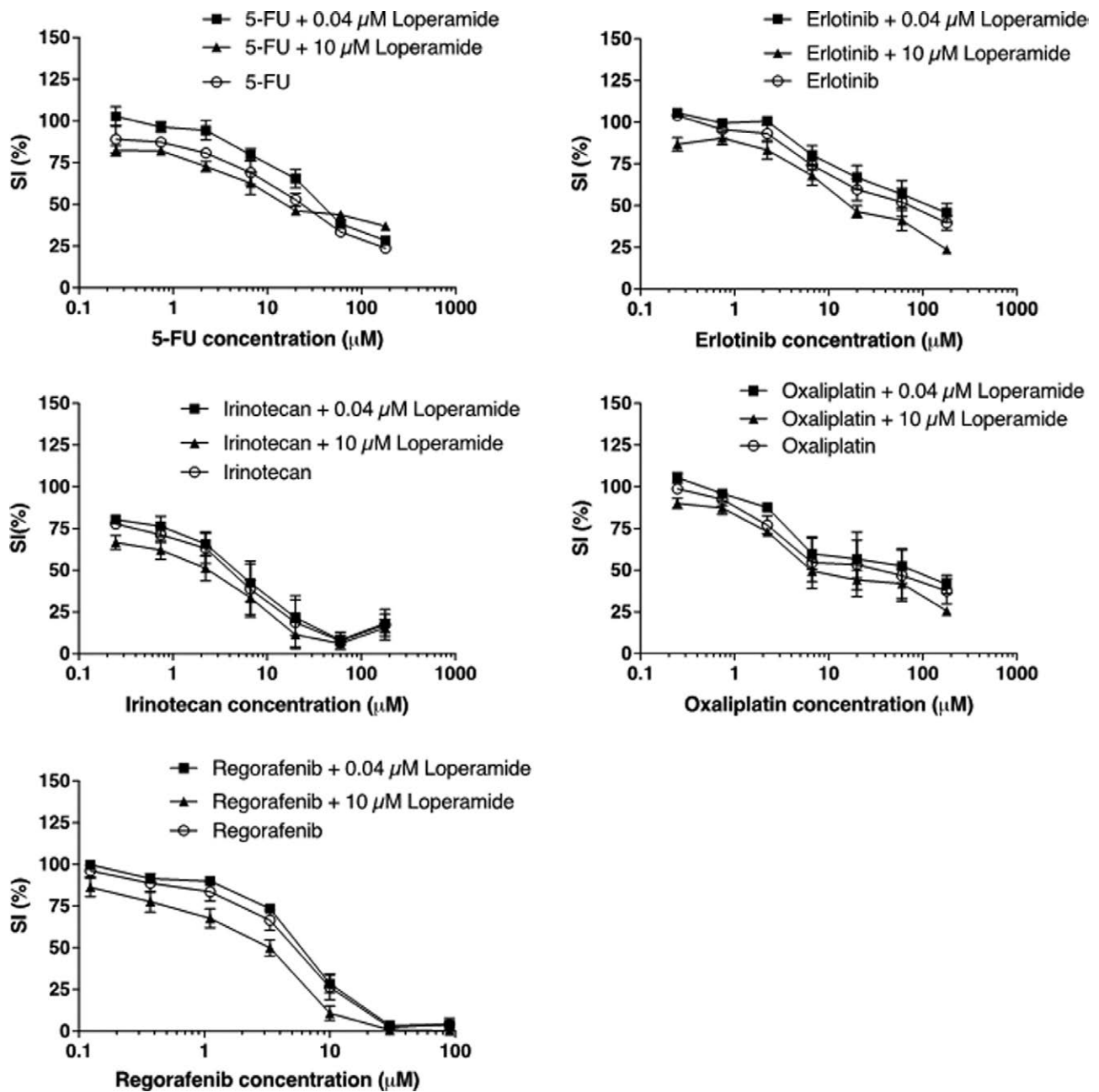
Interaction scores for all pairwise interactions indicated and screened for in the HCT116 wt cells. Bonferroni adjusted antagonism and synergy for each pairwise drug combinations are reported as column deflections to the left and right, respectively, on the interaction score scale. Results are presented as mean values  $\pm$  SEM for three independent experiments, each performed in quadruplicates. \* $P < 0.05$ , \*\* $P < 0.001$  calculated using one-sample *t*-test after subtracting antagonism from synergy.

whether this was a more general phenomenon for other protein kinase inhibitors in clinical use. Figure 5 shows the interaction data for simvastatin with crizotinib, gefitinib, imatinib, lapatinib, nintedanib, sorafenib or vemurafenib. Simvastatin was seemingly synergistic with most kinase inhibitors, most pronounced and statistically significant

with lapatinib and vemurafenib. These data suggest that simvastatin might have a class-effect interaction with kinase inhibitors.

Based on the interaction results for simvastatin in the HCT116wt cell line model, we also investigated its effect

Fig. 3



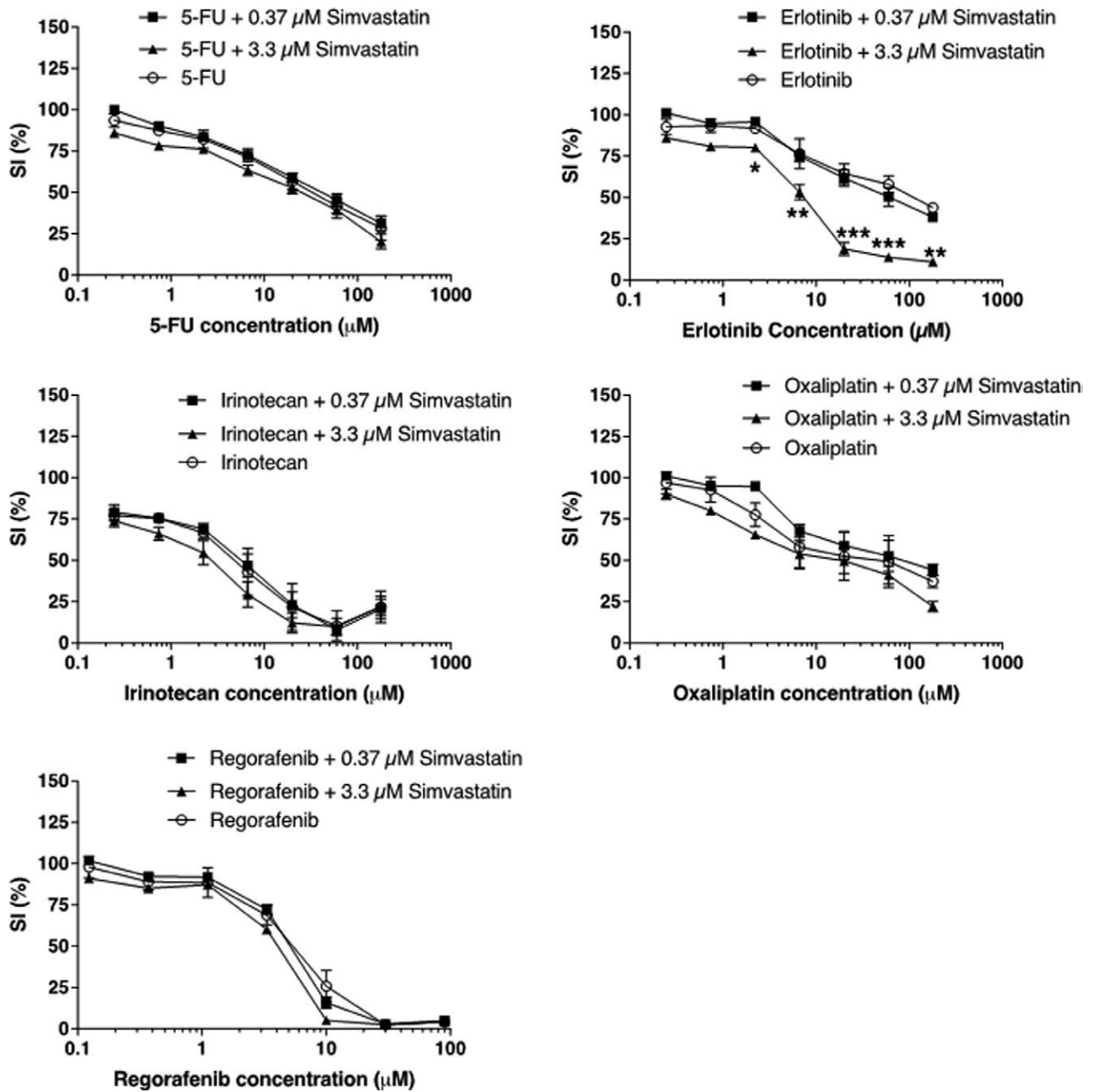
Effect of loperamide at 0.04 or 10  $\mu\text{M}$  on the concentration-response curves for the anticancer drugs indicated in HCT116 wt colon cancer cells. Cell survival was assessed after 72 h of continuous drug exposure and is expressed as survival index (SI) in per cent of unexposed control wells. Results are presented as means  $\pm$  SEM of three independent experiments, each performed in quadruplicates.

in primary cultures of tumor cells from patients with colorectal cancer, a nonproliferative tumor cell model. Simvastatin showed some but less effect alone in this compared with the cell line model (Fig. 6a). Combined with irinotecan there was a trend towards antagonism (Fig. 6b) whereas the very modest effect of erlotinib alone was essentially unaffected by simvastatin at 3.3  $\mu\text{M}$  (Fig. 6c).

## Discussion

All drugs tested except for loperamide and simvastatin were without effect on HCT116wt cells even at or very much higher concentrations than those achievable in patients. Thus, most of these commonly used concomitant drugs are expected to be without effect on tumor cell growth and viability. There is some support that this conclusion apply in the clinic from registry-based

Fig. 4



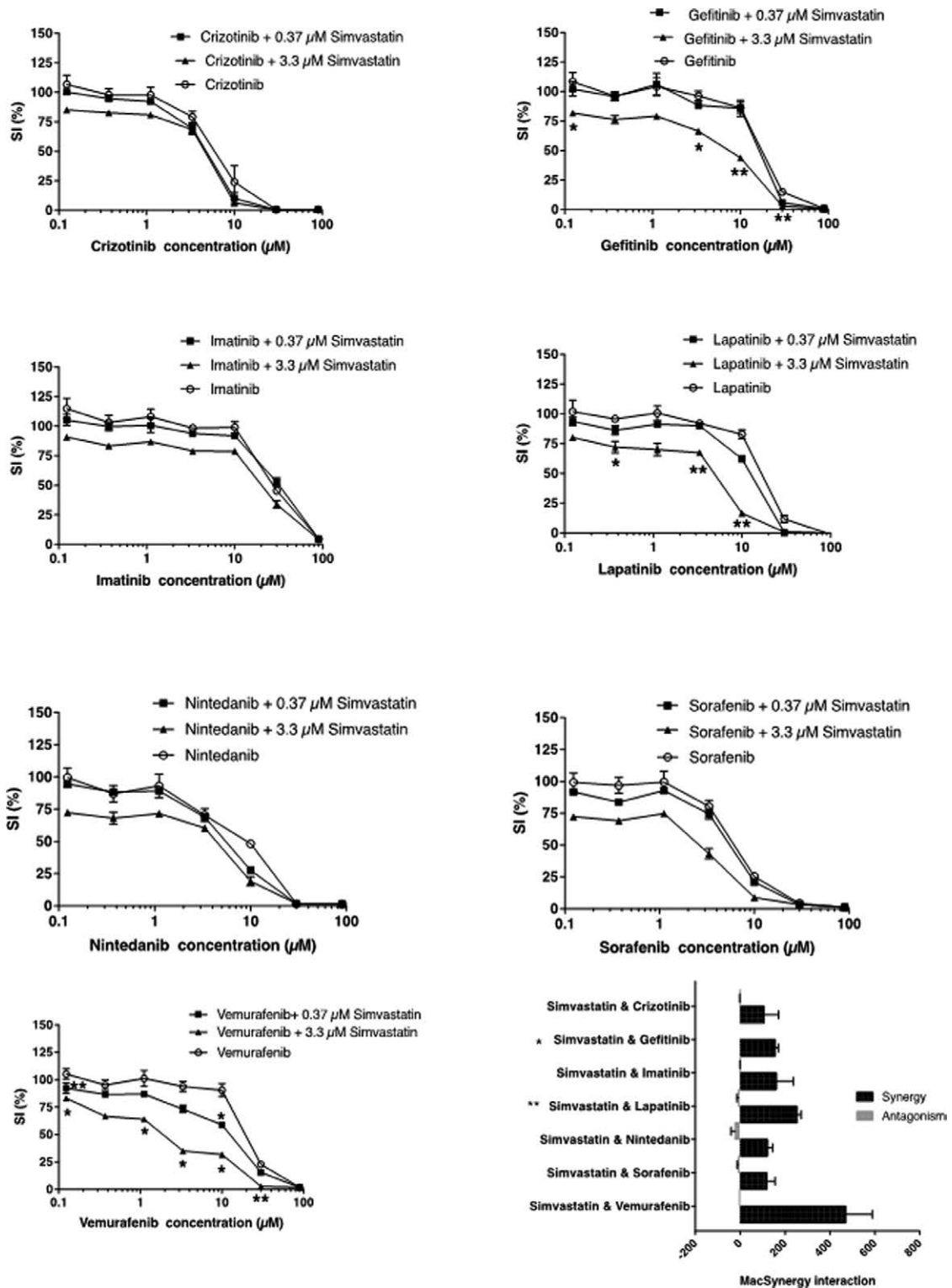
Effect of simvastatin at 0.37 or 3.3  $\mu\text{M}$  on the concentration-response curves for the anticancer drugs indicated in HCT116 wt colon cancer cells. Cell survival was assessed after 72 h of continuous drug exposure and is expressed as survival index (SI) in per cent of unexposed control wells. Results are presented as means  $\pm$  SEM of three independent experiments, each performed in quadruplicates. \* $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$  versus anticancer drug alone calculated using paired Student's *t*-test.

epidemiological data on concurrent new drug prescriptions in patients with early breast cancer [11]. However, simvastatin and loperamide showed cytotoxic effects in HCT116wt cells at concentrations not too far from those achievable in patients. A similar effect for simvastatin was also observed in primary cultures of tumor

cells from patients with colorectal cancer, although these cells were somewhat more resistant (this study).

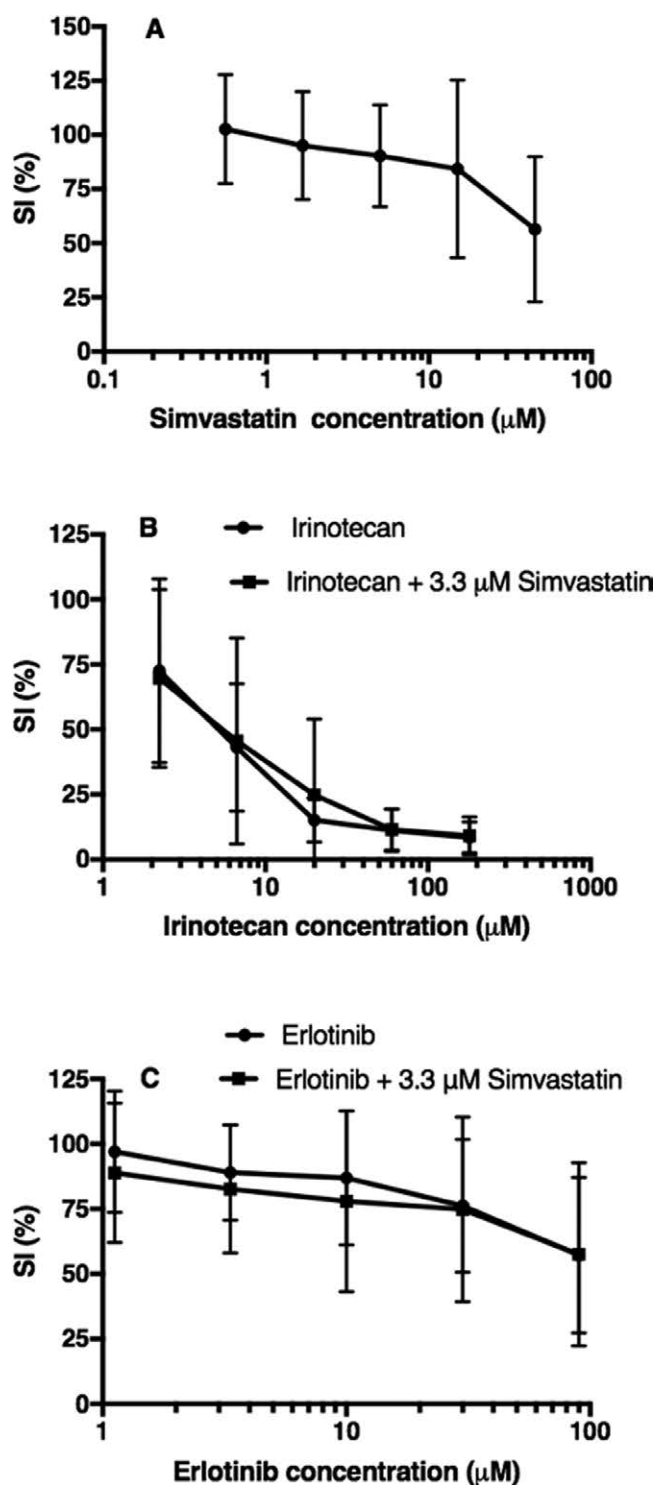
Cytotoxic effects of simvastatin at concentrations close to those achievable in patients have previously been observed in several human cell lines representing

Fig. 5



Effect of simvastatin at 0.37 or 3.3 μM on the concentration-response curves for the protein kinase inhibitors indicated in HCT116 wt colon cancer cells. Cell survival was assessed after 72 h of continuous drug exposure and is expressed as survival index (SI) in per cent of unexposed control wells. Results are presented as means ± SEM of three independent experiments, each performed in quadruplicates. \**P* < 0.05, \*\**P* < 0.001 versus protein kinase inhibitor alone calculated using paired Student's *t*-test. Right lower panel shows the Bonferroni adjusted interaction patterns similarly as in Fig. 2 and with levels of statistical inference as above.

Fig. 6



Effect of simvastatin alone (a) and the effect of simvastatin at 3.3  $\mu\text{M}$  on the concentration-response curves for irinotecan (b) or erlotinib (c) in tumor cells from patients with colorectal cancer. Cell survival was assessed after 72 h of continuous drug exposure and is expressed as survival index (SI) in per cent of unexposed control wells. Results are presented as means  $\pm$  SD of 26 patient samples.

different tumor types, this with cells grown in 2-D monolayers as well as 3-D spheroids [12–21]. Interestingly, the

growth-inhibiting effect was observed to be considerably less in nonmalignant cells indicating some selectivity for



a simvastatin effect in tumors compared to normal cells [17–19, 21]. This could perhaps be related to differences between tumor and normal cell in the mevalonate pathway but may also be associated with differences in cell proliferation rates *in vitro*. Thus, in general, simvastatin was more potent *in vitro* in 2-D monolayer cell culture models allowing for rapid cell proliferation compared to less proliferative 3-D spheroid models with identical cell lines [12,16]. This is in line with the observation in the present study of less effect of simvastatin in primary cultures of tumor cells from patients with colorectal cancer, a model in which the tumor cells show little or no proliferation [10].

Anticancer effects of simvastatin have also been observed in xenograft tumor models *in vivo* [14,18,19,22,23] and there are some indirect epidemiological and clinical trial data in support of a beneficial effect from the concomitant use of simvastatin in early-stage breast cancer patients [11,24]. On the other hand, simvastatin has also been observed to stimulate tumor growth *in vitro* and *in vivo* [25]. Overall, there seems to be a case for further exploration of simvastatin as an anticancer drug.

Loperamide, used as an opioid receptor agonist in the present study, was recently reported to have antiproliferative and apoptosis-inducing effects in several human and canine tumor cell lines and at concentrations very similar to that producing proliferation inhibition in the HCT116wt cells investigated in the current study [26,27]. Although loperamide based on these findings has been suggested to be a potential anticancer drug, its low bioavailability resulting in plasma concentration considerably lower than those inhibiting cell proliferation and inducing apoptosis seemingly preclude such use of loperamide. Morphine, extensively used to treat cancer pain, on the other hand, may reach plasma concentrations more closely to those showing tumor cell proliferation inhibition *in vitro* [28]. However, to our knowledge, there is no report on tumor remission induced by morphine in cancer patients.

We performed the interaction analyses over a wide range of drug concentrations. Using this approach in the present study it became evident that for most drugs antagonistic as well as synergistic interactions between pairs of drugs are observed and depend on drug concentrations (Fig. 2). Thus, it is mostly difficult to categorically claim general synergy or antagonism between drugs and such claims only apply within specified concentration ranges. It also means that findings of, for example, synergy *in vitro* will be difficult to translate to the *in vivo* situation unless there is a very robust pattern of synergy covering broad ranges of drug concentrations.

Based on the net interaction scores metformin showed a pattern of antagonism with most cancer drugs, although not statistically significant. Furthermore, based on

concentration-response curves with metformin at a concentration selected to reflect achievable plasma concentrations or a high concentration that tended to show some activity on its own, it was obvious that the antagonism observed was not produced at these concentrations.

In contrast, loperamide showed a more mixed interaction pattern dominated by antagonism, statistically significant with erlotinib, but synergistic with regorafenib, although not statistically significant. Interestingly, at a loperamide concentration showing some minor cytotoxic effect on its own, that is, 10  $\mu$ M, there were trends to synergy with erlotinib as well as regorafenib, again pointing to the importance of concluding on drug interactions within a context of drug concentrations. Loperamide at 20  $\mu$ M was recently found to act synergistically with doxorubicin *in vitro*, a finding aligning reasonably well with our findings (Fig. 3) [27,29]. Described to be a substrate for P-glycoprotein, and thus to increase the cellular uptake of doxorubicin, there is plenty of room for clinically relevant drug interactions affecting the bioavailability of oral drugs in patients on high dose loperamide.

Simvastatin showed a more homogenous interaction pattern dominated by synergy, especially so in this study when combined with protein kinase inhibitors (Figs. 4 and 5). Synergistic interactions between simvastatin and various anticancer drugs have been observed before, *in vitro* as well as in tumor models in mice [13,14,20,21]. Synergistic interaction *in vitro* is seemingly related to the mechanism of action of the anticancer drug as well as to characteristics of the tumor model with synergy observed in low proliferative nutrient deprived tumor cells [12].

Based on the previous preclinical observations of beneficial interactions between anticancer drugs and simvastatin several clinical trials have evaluated this strategy in randomized as well as noncomparative settings. In colorectal cancer simvastatin combined with standard oxaliplatin or irinotecan-based combination chemotherapy or EGFR antibody did neither add significant efficacy nor toxicity to the anticancer drugs [30–34]. Similar observations have also been made in clinical trials in gastric and pancreatic cancer when combining simvastatin with standard chemotherapy for these cancer types [35,36].

For the most interesting combinations indicated from our study, that is, simvastatin combined with protein kinase inhibitors, a small randomized trial showed a trend towards superiority for simvastatin combined with gefitinib but no effect when combined with afatinib in nonsmall cell lung cancer, in both cases without obvious signs of more toxicity [37,38].

Overall, the quite large numbers of preclinical observations of beneficial interactions between simvastatin and different anticancer drugs have not yet materialized in clinical benefit as investigated in several but rather small

and differently designed clinical trials. This illustrates the difficult step to translate findings of drug interactions *in vitro* to the clinical situation because the interactions seemingly depend on, for example, drug concentrations and tumor cell properties, as illustrated in this and a previous study from our lab [12]

The present study has several strengths. Interactions between a quite large number of drugs, carefully selected to be clinically relevant, especially for the treatment of colorectal cancer, were tested over broad concentrations ranges allowing for assessment of drug concentration-dependent interactions and with key findings further analyzed for other drugs and in a model system established to be clinically relevant, that is, primary cultures of patient tumor cells.

However, only one human tumor cell line to represent colon cancer was investigated and preferably other cell lines from this tumor type should have been included and also contrasted to those derived from other cancer diagnoses to allow for some conclusions on tumor type selectivity and mechanisms. Furthermore, because drug interactions depend on states of cell proliferation and metabolism [12], the inclusion of model systems reflecting different such properties would have been of interest as well as models reflecting nontumor normal cells for assessment of whether interactions observed show selectivity for tumor cells. Still, the fact that the present investigation despite its limitations picked up simvastatin as a drug with both anti-cancer effects and interactive potential points to the relevance of the findings.

## Conclusion

Concomitant drugs commonly used in cancer patients do neither have anticancer effects on their own nor are expected to interact at the tumor cell level with anticancer drugs frequently used for the treatment of colorectal cancer. However, opioid agonists and, especially, simvastatin and perhaps also other cholesterol-lowering similar drugs, show interesting effects worthwhile to investigate further. In this perspective, the class-effect interaction indicated between simvastatin and protein kinase inhibitors deserves attention in experimental as well as clinical research. With respect to the latter, one starting point could be registry data research on the efficacy and tolerance of protein kinase inhibitors in cancer patients prescribed or not simvastatin or other similar cholesterol-lowering drugs.

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## Conflicts of interest

There are no conflicts of interest.

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