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Synergy and antagonism between azacitidine and FLT3 inhibitors

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ABSTRACT

Synergetic interactions between drugs can make a drug combination more effective. Alternatively, they may allow to use lower concentrations and thus avoid toxicities or side effects that not only cause discomfort but might also reduce the overall survival. Here, we studied whether synergy exists between agents that are used for treatment of acute myeloid leukaemia (AML). Azacitidine is a demethylation agent that is used in the treatment of AML patients that are unfit for aggressive chemotherapy. An activating mutation in the FLT3 gene is common in AML patients and in the absence of specific treatment makes prognosis worse. FLT3 inhibitors may be used in such cases. We sought to determine whether combination of azacitidine with a FLT3 inhibitor (gilteritinib, quizartinib, LT-850-166, FN-1501 or FF-10101) displayed synergy or antagonism. To this end, we calculated dose–response matrices of these drug combinations from experiments in human AML cells and subsequently analysed the data using a novel consensus scoring algorithm. The results show that combinations that involved non-covalent FLT3 inhibitors, including the two clinically approved drugs gilteritinib and quizartinib were antagonistic. On the other hand combinations with the covalent inhibitor FF-10101 had some range of concentrations where synergy was observed.

1. Introduction

The majority of patients with Acute myeloid leukaemia (AML) are 60 years of age or older [1], with the estimated overall median age being about 70 years [2,3]. Many of the older patients, or those with co-morbidities are not fit for the standard recommended therapy for adults (3+7 chemotherapy). Azacitidine is a demethylation agent that is approved to be used in such populations, due to its relative efficacy and limited toxicity [4–7]. However, azacitidine as monotherapy is not highly potent. As an example, treatment with azacitidine has only led to 3.8 month improvement in life extension in elderly patients in comparison with standard care [6]. As a demethylation agent, azacitidine prevents the attachment of methyl groups to DNA bases, thereby affecting the transcription of genes that are important for proliferation of the tumour [8]. The exact effect on a specific tumour depend on a multitude of epigenetic factors. Although it is better tolerated than other cytotoxic drugs used in AML, adverse reactions and toxicities are common. In particular, blood toxicities and infections are frequently observed in patients treated by azacitidine [9].

AML patients with mutations in the FLT3 gene, in particular internal tandem duplications (FLT3-ITD) may benefit from therapy aimed at FLT3 inhibition, especially in light of such mutations being activating

[10–12]. Such inhibitors are nowadays included in the treatment of adult patients that carry FLT3-ITD mutations and are fit to receive chemotherapy, but are not routinely prescribed to treat childhood-AML or elderly patients. Gilteritinib is the most commonly prescribed specific FLT3-inhibitor. It is effective but subject to development of resistance (vide infra). Liver and blood toxicities are associated with gilteritinib therapy as is a certain brain syndrome known as posterior reversible encephalopathy syndrome (PRES) [9]. Overall, both azacitidine and FLT3 inhibitors are effective but subject to resistance and dose-related toxicity that limit their usability in a clinical setting. In particular, resistance to FLT3 inhibitors limits the efficacy of such targeted therapy [12–14].

Two strategies were studied by us in order to postpone the emergence of resistance. The first was rotating between two inhibitors, switching from one to another before resistance was shown to take place [15–17]. This strategy has the advantage that only one drug is used at a time, thus limiting potential toxicity. Success, however, has shown to be modest and depended on the resistance mechanism. Another option is the use of combination therapies, with the hope that, if resistance to one drug occurs, the other drug will still be effective [18,19]. Combination therapies can be highly effective in postponing resistance but there is a risk for higher toxicity as well.

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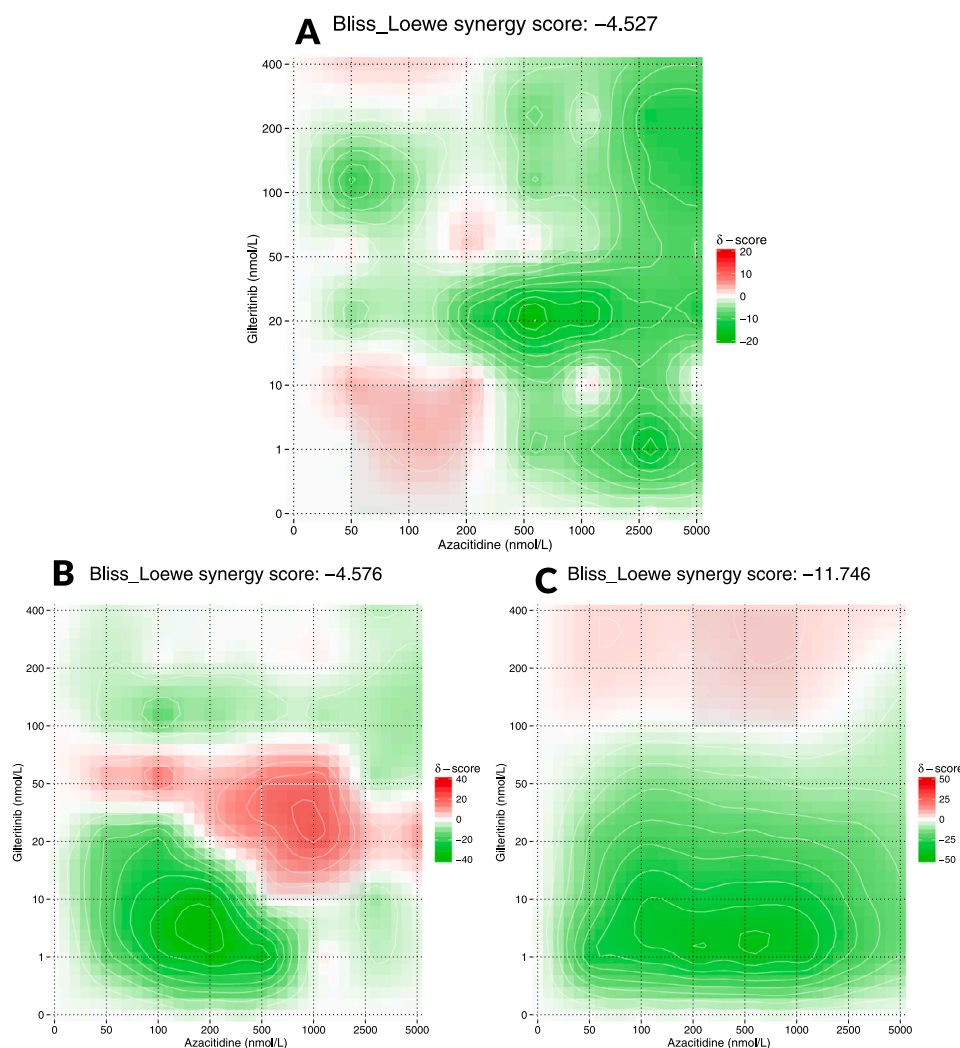


Fig. 1. The drug interaction landscape between azacitidine and gilteritinib based on the Bliss/Loewe reference model on the cell lines MOLM-13 (A), MOLM-14 (B) and MV4-11 (C). The azacitidine concentration is shown on the X-axis and that of gilteritinib on the Y-axis. Red and green colours refer to synergy and antagonism, respectively.

Combination therapies can be synergistic, antagonistic or non-interfering (neither synergistic nor antagonistic). It is often desired to have a synergistic effect. It can be expected that, if the combination is antagonistic, the patients would benefit more from a higher dose of a single drug than from a combination of two. Synergism, on the other hand, can have two positive effects. Firstly, the drug combination can be more effective than a single drug, leading to a more rapid response. Alternatively, doses can be reduced which will lead to better tolerability. This is especially important in frail populations, such as elderly or paediatric patients.

The definition of synergy in drug response is not straightforward, however. Three commonly used estimations of synergy are (1) the Highest Single Agent (HSA) model, where an activity is considered to be synergistic if the two drugs together have better effect than the more effective of these (2) Loewe model [20], where the expected response is calculated as if the two drugs are the same compound and synergy is defined as a better response and (3) Bliss model [21], which assumes an independent action of each drug and thus the expected response is a multiplication of the individual ones. There is vast literature about the choice of a correct model to estimate synergy and antagonism (e.g., [22–25]), but no consensus has emerged.

Considering synergy in cancer treatment has proven to be challenging. Recent reviews considered this issue in-depth [26,27]. Considering AML in particular, Jafari et al. used an approach that combined medical informatics to predict synergistic treatment [28]. Some predictions

were validated in cell models. The authors however noted that their combinations do not take into account specific genetically predefined subsets of AML (e.g., FLT3-mutations). Chory et al. studied multiple drug combinations experimentally, considering different biological mechanisms of drugs [29], but excluding targeted therapies at specified populations (such as patients with FLT3-ITD mutations). Their results suggested combinations of a drug that affect the chromatin states with drugs of several other groups. Overall, the aforementioned studies highlight the potential of rational planning of treatment for AML for considering synergy. However, it is yet unknown how particular combinations can be applied to FLT3-ITD and other types of AML with specific mutations.

Here we study potential synergy between azacitidine and five FLT3 inhibitors (approved or under clinical development): quizartinib, gilteritinib, LT-850-166, FN-1501 and FF-10101. We examine five different agents since each has its own specificities and effects. To ensure that the results are representative we use three AML cell lines that express FLT3-ITD mutants and are sensitive to azacitidine and the FLT3 inhibitors. To determine synergy and antagonism for each combination and cell line, we employ a recently developed consensus method that takes into account HSA, Bliss and Loewe scores (termed Bliss/Loewe [30]) while we also calculated the latter two separately. The consensus scoring strives to eliminate false positives by applying a more stringent criterion for the expected result. If the expected result is higher for the

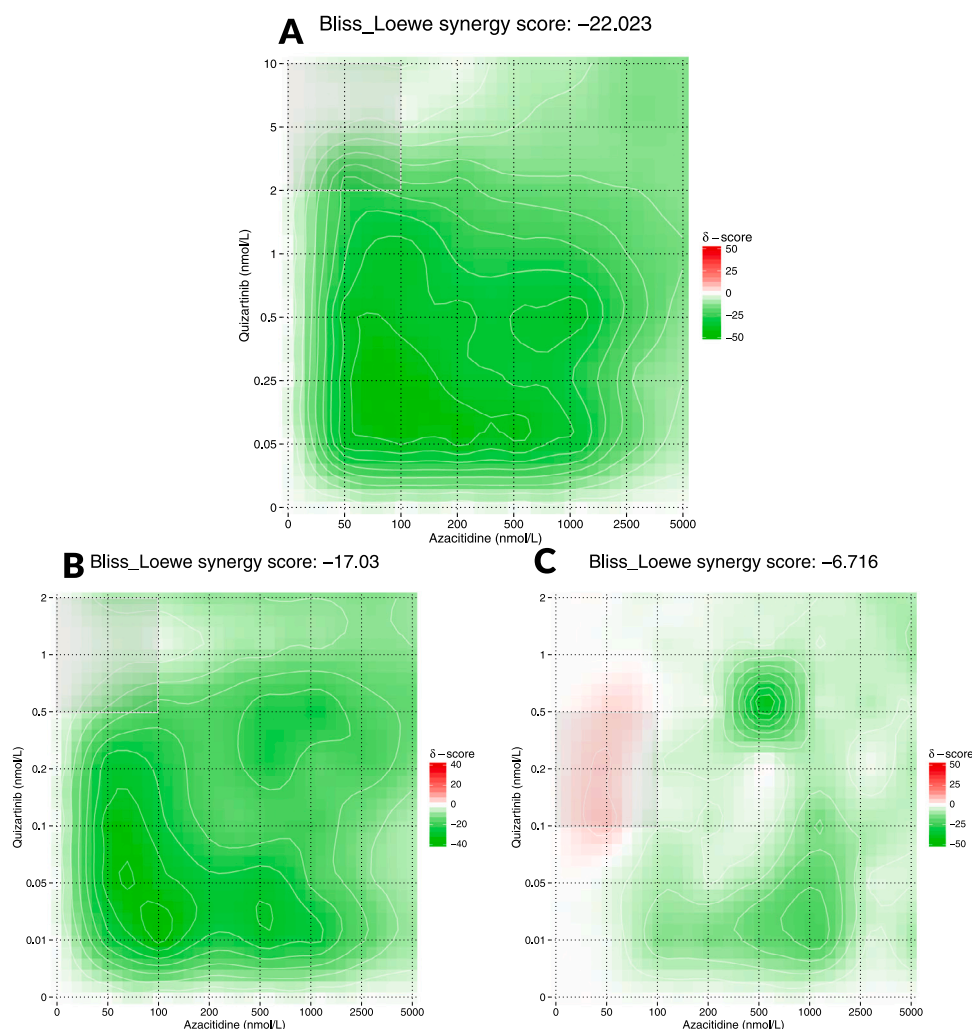


Fig. 2. The drug interaction landscape between azacitidine and quizartinib based on the Bliss/Loewe reference model on the cell lines MOLM-13 (A), MOLM-14 (B) and MV4-11 (C). The azacitidine concentration is shown on the X-axis and that of quizartinib on the Y-axis. Red and green colours refer to synergy and antagonism, respectively.

Bliss model, the Bliss model is used in the consensus scoring, and vice-versa for the Loewe model (the expected value for the HSA model is never higher than for the other two).

2. Results

Synergy analysis is presented as Bliss/Lowe consensus heat maps in Figs. 1–5. In these figures, a positive score (red colour) displays synergy, a negative score (green colour) represents antagonism and an intermediate score (close to zero, white) represent non-interfering effect without synergy or antagonism.

2.1. Azacitidine combined with approved FLT3 inhibitors

We initially tested the combination of azacitidine with each of the two FDA approved specific FLT3 inhibitors, gilteritinib and quizartinib. These inhibitors have some differences in their binding mode (gilteritinib is type 1 and quizartinib is type 2, i.e., they bind different states of the protein) and affinity to other targets.

2.1.1. Azacitidine combined with gilteritinib

Synergy analysis for the combination of azacitidine and gilteritinib is presented in Fig. 1. Individual scores (Bliss and Loewe) are shown in Figures S1–S3. The synergy landscapes show antagonism throughout most of the concentration ranges for all cell lines. There is an area in

the middle of the concentration range where the interaction is clearly synergistic in MOLM-14 cells but not in the other cell lines. Overall, the combination is antagonistic.

2.1.2. Azacitidine combined with quizartinib

The heatmaps for the combination between azacitidine and quizartinib are shown in Fig. 2. Individual scores (Bliss and Loewe) are shown in Figures S4–S6. The heatmaps are characterised by negative values almost everywhere and in all cell lines, i.e., this combination is antagonistic. Comparing quizartinib to gilteritinib, it can be seen that, while the combination of both drugs with azacitidine is antagonistic, the results are clearly worse with quizartinib.

2.2. Azacitidine combined with novel non-covalent FLT3 inhibitors

Following the examination of combinations with approved inhibitors, we set to examine the combinations of azacitidine with two novel FLT3 inhibitors that are currently under development and which bind FLT3 non-covalently. These are the potent inhibitor LT-850-166 with high affinity to FLT3 and several mutants that confer resistance to gilteritinib and/or quizartinib [31] and FN-1501 which is a multiple kinase inhibitor with potency against FLT3 and CDK2, CDK4 and CDK6 [32]. FN-1501 is under clinical development. It showed anti-tumour activity in patients, and its safety and tolerability were considered reasonable [33,34]

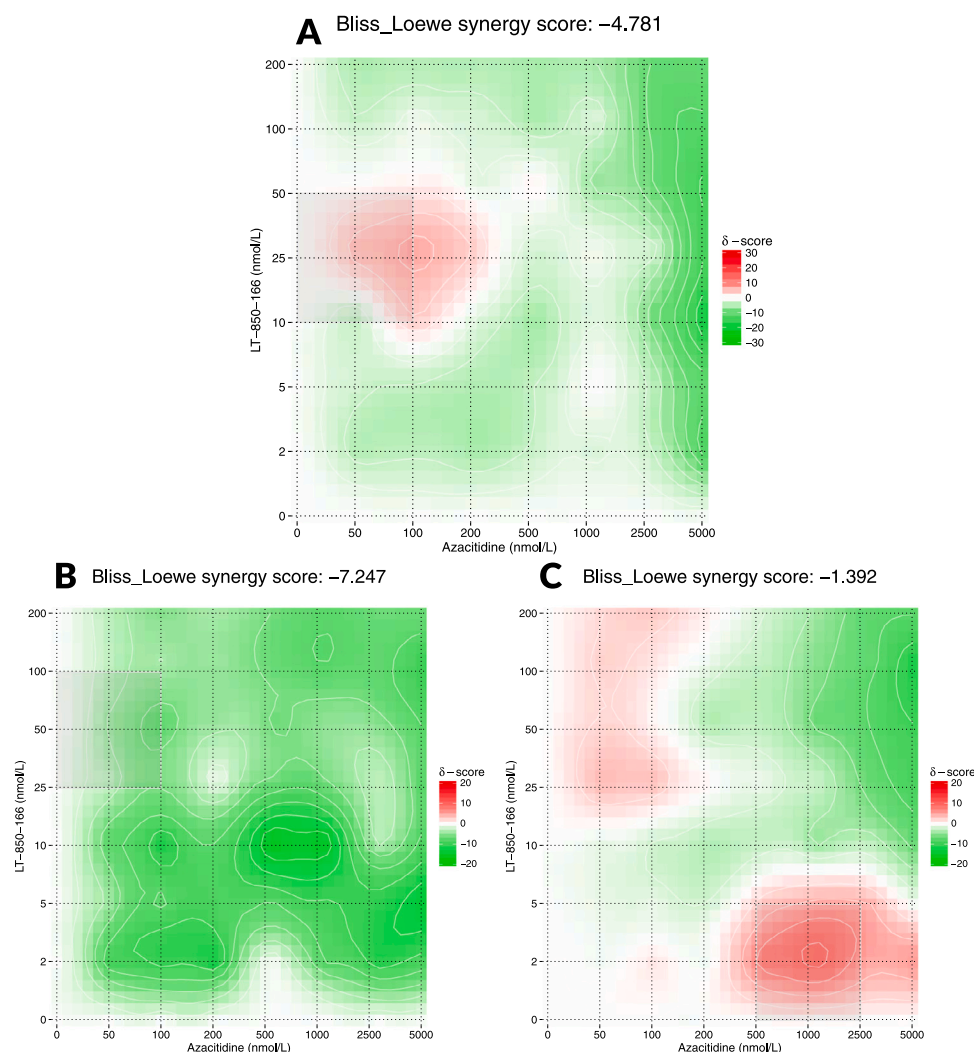


Fig. 3. The drug interaction landscape between azacitidine and LT-850-166 based on the Bliss/Loewe reference model on the cell lines MOLM-13 (A), MOLM-14 (B) and MV4-11 (C). The azacitidine concentration is shown on the X-axis and that of LT-850-166 on the Y-axis. Red and green colours refer to synergy and antagonism, respectively.

2.2.1. Azacitidine combined with LT-850-166

The heatmaps for the combination between azacitidine and LT-850-166 are shown in Fig. 3. Individual scores (Bliss and Loewe) are shown in Figures S7–S9. This combination is overall antagonistic. In similarity with gilteritinib, in individual cell lines there are patches of synergy but these do not agree between different cell lines. Experiments with MOLM-14 showed antagonism all across the heatmap.

2.2.2. Azacitidine combined with FN-1501

Synergy analysis for the combination of azacitidine and gilteritinib is presented in Fig. 4. Individual scores (Bliss and Loewe) are shown in Figures S10–S12. Similar to quizartinib, the interaction between FN-1501 and azacitidine is antagonistic across all cell lines and almost all concentrations.

2.3. Azacitidine combined with the covalent inhibitor FF-10101

Lastly, we set to examine the combination of azacitidine with the first-in-class FLT3 covalent inhibitor currently under clinical development [35]. Synergy analysis for this combination is presented in Fig. 5. Individual scores (Bliss and Loewe) are shown in Figures S13–S15. The results are somewhat more encouraging than those presented for the non-covalent inhibitors. There is a stretch of red-coloured area in the heatmaps in all cell lines, showing clear synergy, at the high-end of the

tested FF-10101 concentrations (~5 nM in MOLM-13 and MV4-11 and ~2 nM in MOLM-14 cells), and spanning a large range of azacitidine concentrations.

3. Discussion

In this study, we set to examine if azacitidine, a demethylation agent used for treatment of AML, synergises with FLT3 inhibitors in FLT3⁺-AML cells. Azacitidine leads to reduction in DNA methylation of many genes and thus affects multiple pathways, but the exact mechanisms by which it leads to clinical improvement are not known in detail. Synergy in cancer therapy is mostly the result of drugs that limit tumour growth by multiple mechanisms (see [36] for an exception) and is often concentration-dependent. In low concentrations, drugs will only affect their intended targets (sometimes only partially) whereas in higher concentrations they will also interact with other proteins or biogenic molecules. In many cases, synergy will be observed somewhere along a combined range of drug concentrations. Antagonism between drugs can be the result of competition for the same target, opposite effects (e.g., one agent inhibits a certain pathway and the other activates it) or toxicity to healthy cells. The latter was not considered in this study, whereas the former (opposite effect) is more likely since anti-cancer drugs often affect multiple pathways in an intricate manner.

The main finding from this study is that no synergy was observed in all cell lines between azacitidine and the non-covalent FLT3 inhibitors.

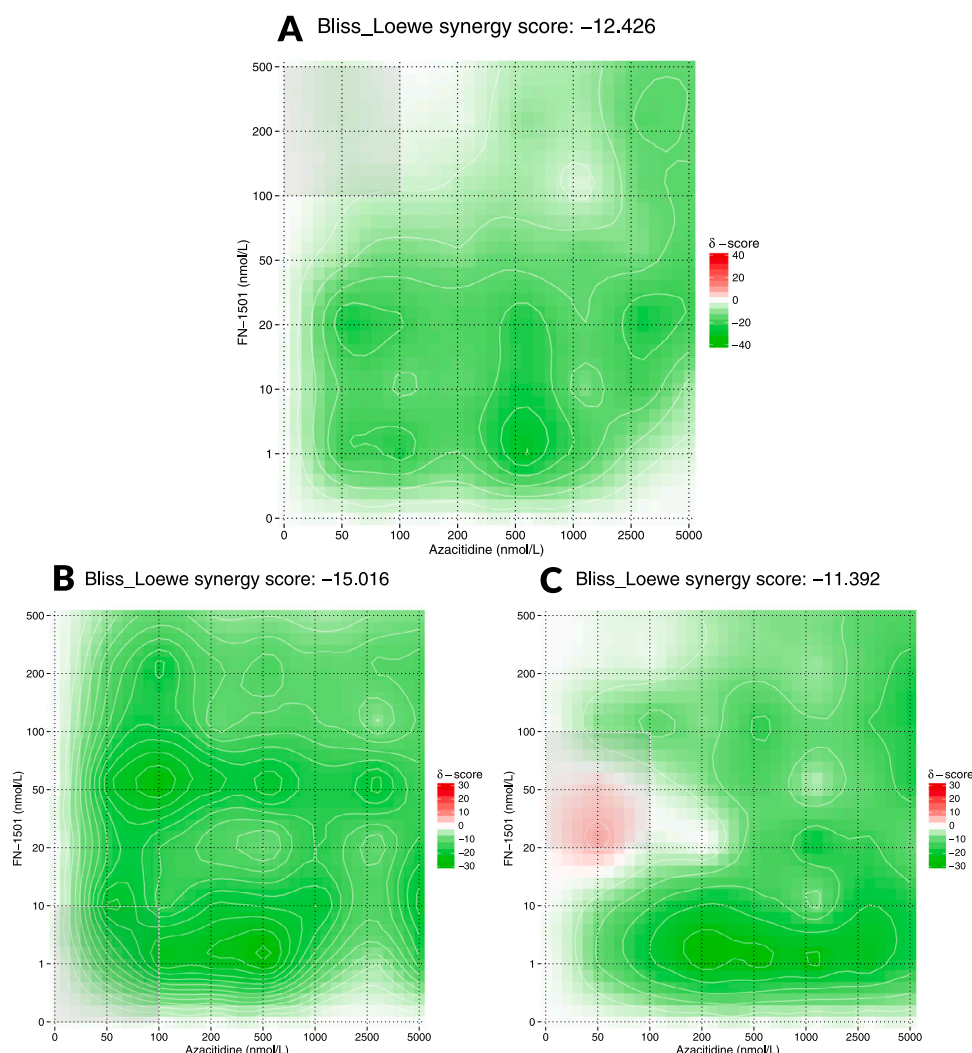


Fig. 4. The drug interaction landscape between azacitidine and FN-1501 based on the Bliss/Loewe reference model on the cell lines MOLM-13 (A), MOLM-14 (B) and MV4-11 (C). The azacitidine concentration is shown on the X-axis and that of FN-1501 on the Y-axis. Red and green colours refer to synergy and antagonism, respectively.

Rather, these combinations were mostly antagonistic. Interestingly, during the work on this manuscript the results of a phase III trial were reported where elderly patients were treated with azacitidine and gilteritinib versus azacitidine as monotherapy [37]. The combination was definitely not better and was even worse than monotherapy for long-term overall survival, which is in line with the cell studies performed in this work (that showed antagonism between the drugs). We find it encouraging that studies in cell lines might be predictive of a clinical outcome.

In terms of the methodology, it was necessary to consider multiple cell lines and scoring methods, to get relevant insights. In most cases, the Loewe and Bliss scores agreed with each other, but there were few cases where, for some of the range, Loewe score showed synergy and Bliss score showed antagonism. As the correct choice of the method is not clear, we opted for the consensus Bliss/Loewe score that also includes the HSA model and have used this in all calculations. For example, strong synergy was shown for the combination of gilteritinib and azacitidine in MOLM-13 cells at 50 nM gilteritinib and 5 μ M azacitidine when the Loewe score was used, but antagonism was evident when the Bliss score was used (Figure S1). Moreover, the same combination of inhibitors and concentrations was antagonistic in MV4-11 cells. Considering the different methods to calculate synergy, the Loewe

calculation in general resulted in higher synergy values. Importantly, considering the three cell lines together, our main conclusion, i.e., the only FF-10101 shows synergy, remains valid irrespective of the choice of scoring model.

FF-10101 is a covalent inhibitor and thus its binding mode is different than that of other FLT3 inhibitors as it relies on binding to Cys⁶⁹⁵ that is otherwise not involved in inhibitor binding. Gilteritinib, for example, has high affinities to FLT3 and AXL; these proteins have 40% similarity and 24% identity in the region that includes Cys⁶⁹⁵ (residues 544–707 in FLT3) but the corresponding residue in AXL is Lys⁶²⁴. A cysteine residue corresponding to Cys⁶⁹⁵ is not found in ALK, RET, ROS and LTK which are the proteins for which gilteritinib is known to have high affinity (data from BindingDB, <https://www.bindingdb.org> [38]), and it is thus not expected that FF-10101 will bind any of these. Therefore FF-10101 is expected to be highly specific. This specificity might contribute to the synergy that is observed between FF-10101 and azacitidine at the high end of the concentration range of the FLT3 inhibitor, where FLT3 is inhibited almost completely. With the other inhibitors, when the FLT3 inhibitor concentration is high, additional proteins are also inhibited which likely affects the efficacy of azacitidine in a negative way.

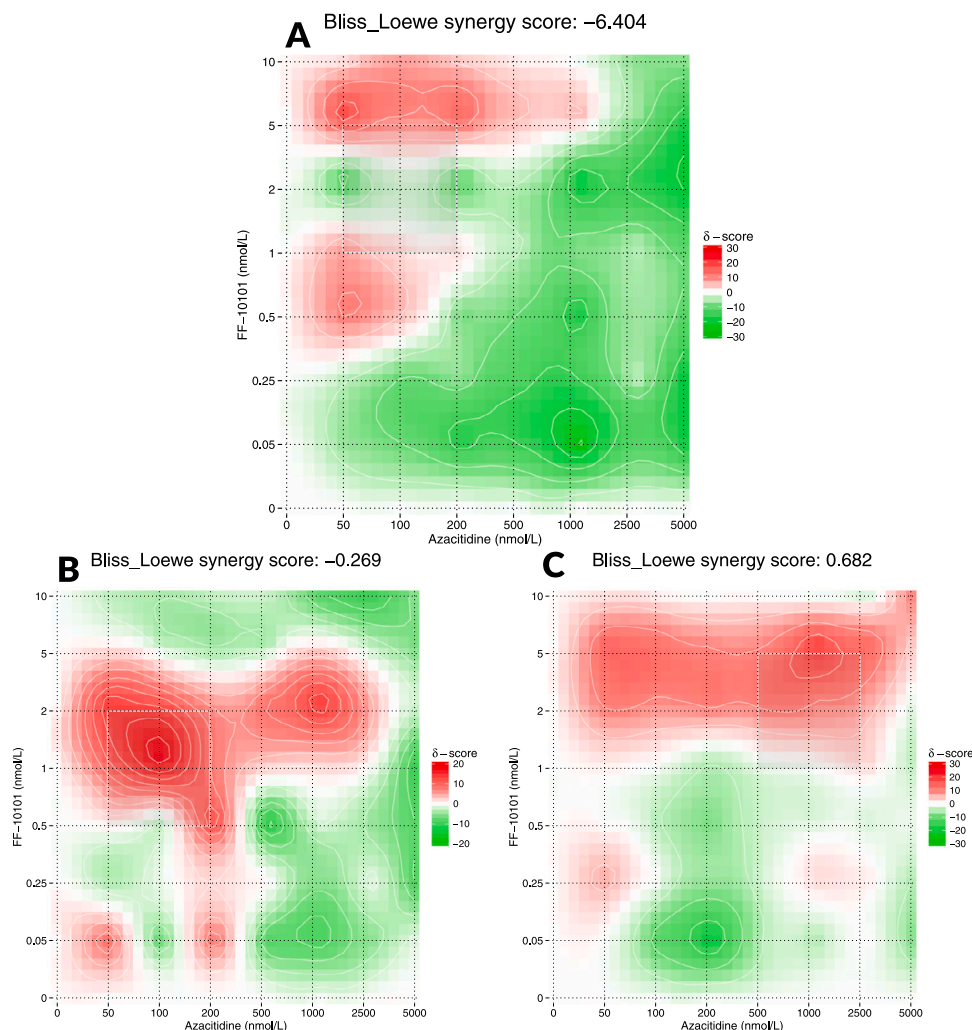


Fig. 5. The drug interaction landscape between azacitidine and FF-10101 based on the Bliss/Loewe reference model on the cell lines MOLM-13 (A), MOLM-14 (B) and MV4-11 (C). The azacitidine concentration is shown on the X-axis and that of FF-10101 on the Y-axis. Red and green colours refer to synergy and antagonism, respectively.

Finally, we suggest that synergy analysis will be part of the pre-clinical evaluation of combination therapy. Prior to starting with animal experiments or human studies, it is beneficial to show that any studied combination is synergistic in the relevant range of concentrations, or at least it is not antagonistic. Synergy studies could also be used to decide on the dosage range for both drugs. Based on this study, a combination of FF-10101 and azacitidine has the potential to succeed where a similar combination with gilteritinib was not beneficial.

4. Materials and methods

4.1. Regents

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was purchased from Promega Biotech AB.

4.2. Inhibitors

Azacitidine, FN-1501, gilteritinib and quizartinib were purchased from MedChemExpress; LT-850-166 was a generous gift from Prof. Shuai Lu, School of Science, China Pharmaceutical University, Nanjing, PR China. FF-10101 was purchased from Chemtronica AB (Sweden).

4.3. Cell lines and cell culture

AML cell lines, MOLM-13 and MOLM-14 were cultured in RPMI 1640 medium, while the MV4-11 cell line was grown in IMDM medium. All cell culturing media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin G and streptomycin as antibiotics at 37° C in an atmosphere of 5% CO₂ in a humidified incubator.

4.4. Dose response curves

Dose response curves were measured by cell viability assays as described in [19]. Numerical IC₅₀ and IC₉₀ values were calculated by nonlinear best-fit regression analysis using the Prism 8 software (GraphPad, Inc.).

4.5. Analysis of synergy

Azacitidine was combined with different FLT3 inhibitors (FF-10101, FN-1501, gilteritinib, LT-850-166 and quizartinib) and MTS assays were carried out in 96-well plates. Treatment with different combinations of azacitidine (5000 nM, 2500 nM, 1000 nM, 500 nM, 200 nM, 100 nM, 50 nM) and FF-10101 (10 nM, 5 nM, 2 nM, 1 nM, 0.5

nM, 0.25 nM, 0.05 nM) or FN-1501 (500 nM, 200 nM, 100 nM, 50 nM, 20 nM, 10 nM, 1 nM) or gilteritinib (400 nM, 200 nM, 100 nM, 50 nM, 20 nM, 10 nM, 1 nM) or LT-850-166 (200 nM, 100 nM, 50 nM, 25 nM, 10 nM, 5 nM, 2 nM) or quizartinib (2 nM, 1 nM, 0.5 nM, 0.2 nM, 0.1 nM, 0.05 nM, 0.01 nM) was established for 48 h. To clarify, concentration ranges of (2 nM, 1 nM, 0.5 nM, 0.2 nM, 0.1 nM, 0.05 nM, 0.01 nM) were used with for MV4-11 and MOLM-14 and the concentration range of quizartinib for MOLM-13 was (10 nM, 5 nM, 2 nM, 1 nM, 0.5 nM, 0.25 nM, 0.05 nM). We chose IC₉₀ values or slightly higher (see Supporting Information, Table S1) as the highest concentration of FLT3 inhibitors in order to have a wide range of OD values for the MTS assay. It is not meaningful to use even higher values as in this case few if any cells survive when using combinations. We chose 5 μ M (around IC₅₀) value for azacitidine (see Supporting Information, Table S1) as the highest concentration of azacitidine. Since FLT3 inhibitors already were at a very wide range of concentrations, 5 μ M was high enough for the combination assay without affecting the OD. Data from cell cytotoxicity assays were analysed with a web application, SynergyFinder 3.0 [30](<https://synergyfinder.fimm.fi/>).

4.6. Synergy score

There are multiple reference models used to assess synergy and analyse interactions of anti-cancer drugs [39–41]. To evaluate interactions between drugs as reliably as possible, we analysed the data from combination studies with three majorly used reference models: Bliss, Loewe and a novel synergy scoring method called Bliss/Loewe consensus that also includes HSA. These models were formulated with specific empirical or biological assumptions. The synergy score (δ) was used to evaluate the efficacy of the combination. δ is interpreted as follows:

$\delta < -10$: the interaction between two drugs is likely to be antagonistic;

$-10 < \delta < 10$: the interaction between two drugs is likely to be non-interfering;

$\delta > 10$: the interaction between two drugs is likely to be synergistic [42].

CRediT authorship contribution statement

Jingmei Yang: Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Ran Friedman:** Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at <https://doi.org/10.1016/j.compbiomed.2023.107889>.

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