Better to be alone than in bad company: The antagonistic effect of cisplatin and crizotinib combination therapy in non-small cell lung cancer

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AIM
To investigate the potential benefit of combining the cMET inhibitor crizotinib and cisplatin we performed in vitro combination studies.

METHODS
We tested three different treatment schemes in four non-small cell lung cancer (NSCLC) cell lines with a different cMET/epidermal growth factor receptor genetic background by means of the sulfurhodamine B assay and performed analysis with CalcuSyn.

RESULTS
All treatment schemes showed an antagonistic effect in all cell lines, independent of the cMET status. Despite their different genetic backgrounds, all cell lines (EBC-1,
HCC827, H1975 and LUDLU-1) showed antagonistic combination indexes ranging from 1.3-2.7. These results were independent of the treatment schedule.

CONCLUSION
These results discourage further efforts to combine cMET inhibition with cisplatin chemotherapy in NSCLC.

Key words: Non-small cell lung cancer; Combination therapy; Cisplatin; Crizotinib; cMET

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Core tip: Targeted therapies are a valuable treatment option in non-small cell lung cancer. Several therapies have now been approved like erlotinib and gefitinib for epidermal growth factor receptor - mutant patients and crizotinib for Anaplastic Lymphoma Kinase-rearranged patients. However, resistance against these therapies eventually occurs. Combination therapy might be able to overcome or delay this resistance. Here we investigate the combination of the cMET inhibitor crizotinib with cisplatin in a panel of non-small cell lung cancer (NSCLC) cell lines with different histological and genetic backgrounds. We show that this leads to strong antagonism in all of the used cell lines. Furthermore we also link these results to the earlier in vitro and clinical results of the combination of erlotinib/gefitinib with cisplatin based chemotherapy in NSCLC.

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INTRODUCTION
During the last decade, targeted therapies have revolutionized the treatment for non-small cell lung cancer (NSCLC). Several epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) have been approved for patients with sensitizing mutations in EGFR[4,5]. Furthermore, several cMET inhibitors are currently under development with promising clinical benefit[6-9]. However, only a small percentage of NSCLC patients are eligible for these treatments. Thus, for the majority of NSCLC patients, cisplatin based therapy remains the standard of care treatment in first or later lines, usually in combination with pemetrexed, gemcitabine or a taxane[10-12].

CMET, with its ligand hepatocyte growth factor (HGF), is known to be activated in many tumor types, including NSCLC[10], with cMET amplification recognized as a resistance mechanism during EGFR tyrosine kinase inhibition[11]. The cMET and EGFR signaling pathways are heavily intertwined[12,13], with EGFR activation being sufficient for downstream cMET phosphorylation. The mitogen activated protein kinase (MAPK) dependent activation of cMET by EGFR takes place at different regulatory levels, with cMET transcriptional upregulation, the elongation of cMET half-life and a decrease in cMET-ubiquitylation[12]. Upon binding of HGF, the cMET receptor dimerizes and cross-phosphorylation takes place. This ultimately leads to phosphorylation of the docking sites recruiting proteins involved in the signaling of MAPK cascades, phosphoinositide 3 kinase (PI3K), signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB). Thus activating many oncogenic processes such as migration, invasion, and angiogenesis[14]. Two main cMET aberrations have been described, which can be used to predict sensitivity to cMET therapies: Amplification of the cMET gene[15] and cMET exon 14 skipping[16,17].

Several small molecule inhibitors and monoclonal antibodies inhibiting cMET signaling are currently being investigated in several clinical trials[16,17]. One of these small molecule inhibitors is crizotinib, which was originally developed as a cMET inhibitor[18] but has been approved for treatment of anaplastic lymphoma kinase (ALK)-translocated NSCLC patients[19]. Currently, crizotinib is being investigated in several clinical trials (METROS trial and the NCT02499614) for the treatment of patients with cMET-dependent NSCLC and in other cancer types where patients carry a cMET amplification[16,19].

The combination of a cMET inhibitor and cisplatin has not been investigated in NSCLC patients to date. However, in vitro studies show contradictory results where the outcome is dependent on tumor type and origin. For example, addition of the cMET ligand HGF enhanced cisplatin resistance in seven different NSCLC cell lines. This was explained by the fact that HGF binding induces cMET signaling which led to activation of focal adhesion kinase (FAK). FAK, in turn, suppressed the apoptosis inducing factor (AIF), resulting in a decreased sensitivity towards cisplatin[20]. Therefore, theoretically, inhibition of cMET could possibly result in sensitization towards cisplatin. However, another study in SW620 cells, a KRAS mutated colon cancer cell line, showed that conditioned knock-down of cMET did not influence cisplatin sensitivity[21]. In contrast, ovarian cancer cell lines were sensitized towards cisplatin with the addition of HGF[22], this was established to be linked to the p38-MAPK signaling of cMET[23]. HGF pretreatment of these cells decreased the transcription of protein phosphatase 2A, thus increasing the effect of cisplatin[24].

Given the contradictory results in previous studies, more studies were warranted. Therefore, we investigated whether a combination of these compounds could result in a synergistic treatment effect in NSCLC cell lines with different cMET and EGFR genetic backgrounds.
MATERIALS AND METHODS

Cell lines and reagents

Four NSCLC cell lines were included in this study. The HCC827 and H1975 cell lines were purchased from the American Type Culture Collection (ATCC), the EBC-1 cell line from the Japanese Collection of Research Bioresources (JCRB, Japan) and the LUDLU-1 cell line from the European Collection of Authenticated Cell Cultures (ECACC) (Figure 1 and Table 1). The EBC-1 cell line was cultured in DMEM (Invitrogen, Merelbeke, Belgium) supplemented with 10% FBS, 1% penicillin/streptomycin, L-glutamine (2 mmol/L) and sodium pyruvate (1 mmol/L). The HCC827, H1975 and LUDLU-1 cell lines were cultured in RPMI1640 (Invitrogen) supplemented with 10% FBS, 1% penicillin/streptomycin, L-glutamine (2 mmol/L) and sodium pyruvate (1 mmol/L). Cultures were incubated at 37 °C under an atmosphere of 5% CO2. The HCC827 cell line harbors an exon 19 deletion in the ErbB1 gene [25], while the H1975 cell line has L858R and T790M mutations in the ErbB1 gene [26]. The EBC-1 cell line harbors a cMET amplification [27], while the LUDLU-1 is wild-type for both EGFR and cMET (Table 1). All cell lines were wild-type for ALK, free from mycoplasma contamination and STR profiles were checked.

Cisplatin and crizotinib were purchased from Sellck-chem (Huissen, The Netherlands). Cisplatin was dissolved in a sterile 0.9% NaCl solution (Fisher Scientific, Aalst, Belgium), while crizotinib was dissolved in dimethyl-sulfoxide (DMSO). Both were diluted in phosphate buffered saline (PBS) to the desired concentrations. Cells were treated with cisplatin or crizotinib during 72 h. Drug sensitivity is given in µmol/L and given as IC50 ± SEM of 3 separate experiments. EGFR: Epidermal growth factor receptor.

Figure 1  Sensitivity of several non-small cell lung cancer cell lines to cisplatin (A) and crizotinib (B) monotherapy. Cells were exposed to the drugs for 72 h. Cisplatin and crizotinib concentrations are depicted in µmol/L. Values are means of at least 3 separate experiments. The maximal SEM was ± 9%.

Table 1  Cell line properties and drug sensitivity

|          | HCC827 | H1975 | EBC-1 | LUDLU |
|----------|--------|-------|-------|-------|
| Properties | Histology | Adeno Exon 19 deletion | Adeno L858R + T790M | Squamous Wild-type | Squamous Wild-type |
| EGFR-status | Wild-type | Wild-type | Wild-type | Wild-type |
| cMET-status | Wild-type | Wild-type | Amplification | Wild-type |
| Drug sensitivity (µmol/L, IC50 ± SEM) | 8.39 ± 0.36 | 6.10 ± 0.07 | 16.52 ± 0.89 | 3.37 ± 0.19 |
| Cisplatin | 6.05 ± 0.11 | 4.00 ± 0.06 | 0.054 ± 0.002 | 8.12 ± 0.28 |

Cells were treated with cisplatin or crizotinib during 72 h. Drug sensitivity is given in µmol/L and given as IC50 ± SEM of 3 separate experiments. EGFR: Epidermal growth factor receptor.
were investigated: (1) simultaneous exposure to cisplatin and crizotinib for 72 h; (2) cisplatin for 72 h, followed by washing and crizotinib for 72 h; or (3) 72 h of crizotinib followed by washing and cisplatin for 72 h (Table 2).

When crizotinib was used as first drug, the concentration was reduced in three out of the four cell lines, due to the toxic after-effect of this drug.

After treatment, growth inhibition was determined by the sulforhodamine B (SRB) assay, as previously described[28]. In short, the medium was discarded and the cells were fixed with ice cold 10% Trichloric acid (Fisher Scientific, Aalst, Belgium) dissolved in 1% glacial acetic acid (Fisher Scientific, Aalst, Belgium) and read at an optical density (OD) of 540 nm (IMark microplate absorbance reader, Biorad, Nazareth, Belgium)[29].

### Statistical analysis

Each test was performed at least three times, unless otherwise stated. Results are presented as mean ± SEM.

To assess the IC50 value of cisplatin and crizotinib, WinNonlin software was used (Pharsight Corporation, Mountain View, CA, United States). To determine possible synergism between cisplatin and crizotinib, the combination index (CI) was calculated with the CalcuSyn software of Biosoft. This program is based on the method of Chou et al.[30,31] to assess whether a combination of two drugs results in an antagonistic effect (CI > 1.2), an additive effect (0.8 < CI < 1.2) or a synergistic effect (CI < 0.8). This method takes into account the fraction of affected cells of both monotherapies and compares this with the fraction of affected cells of the combination therapies.

### RESULTS

The effects of cisplatin and crizotinib monotherapy were investigated in four NSCLC cell lines (Figure 1). LUDLU-1 cells were most sensitive to cisplatin, followed by the EGFR-mutated H1975 and HCC827 cell lines. As for the cMET amplified EBC-1 cell line, concentrations up to 10 µM cisplatin induced only 30% growth inhibition and the IC50 value was determined by extrapolation (Figure 1).

EBC-1 cells were 74-150 fold more sensitive to crizotinib than the other 3 cell lines, due to the presence of a cMET amplification in these cells. The IC50 values of the HCC827 and LUDLU-1 cell line were determined by extrapolation, with the LUDLU-1 being the most resistant to crizotinib (Figure 1 and Table 1). Based on these results, we decided to use the IC50 and IC0 values of crizotinib during combination treatment (Table 2).

Despite their different genetic backgrounds for cMET and EGFR, all cell lines showed strong antagonism (CI ranging from 1.3 to 2.7) when crizotinib and cisplatin were combined, which was independent of the used treatment schedule (Table 2). This antagonistic effect was visible for all growth inhibition rates of the cells (Figure 2). However, for one treatment condition, i.e., crizotinib followed by cisplatin treatment in the H1975 cell line, an additive effect (CI = 1.0) could be detected. However, this combination only led to 40% growth inhibition at most and needs to be interpreted with caution.

### DISCUSSION

Although both cisplatin and crizotinib are active drugs used in monotherapy for the treatment of various forms of NSCLC, the combination of both compounds was found to be antagonistic, independent of the genetic background of the investigated cell lines.

As described in literature, the high sensitivity of the EBC-1 cell line for crizotinib monotherapy can be explained by its cMET amplification, which is known to confer sensitivity to crizotinib and other cMET small molecule inhibitors[19]. In contrast, the EBC-1 cells were not sensitive to cisplatin, with an IC50 value around 16 µM. Unfortunately, in Table 1, the CI values for crizotinib monotherapy were not provided, making it difficult to compare the effects of cisplatin and crizotinib in the LUDLU-1 cell line. However, in Table 2, the CI values for the combination of cisplatin and crizotinib were provided, allowing us to compare the effects of the two drugs in different cell lines.

### Table 2  Combination indexes for the different non-small cell lung cancer cell lines for the 3 treatment schemes

| Drug scheme | HCC827 | H1975 | EBC-1 | LUDLU-1 |
|-------------|--------|-------|-------|---------|
| Cisplatin + | 3 µmol/L | 1.58 ± 0.10 | 3 µmol/L | 1.94 ± 0.27 | 0.025 µmol/L | 2.08 ± 0.49 | 3 µmol/L | 2.65 ± 0.30 |
| Crizotinib | 5 µmol/L | 1.54 ± 0.15 | 5 µmol/L | 1.93 ± 0.19 | 0.05 µmol/L | 1.42 ± 0.06 | 4 µmol/L | 2.71 ± 0.14 |
| Cisplatin | 3 µmol/L | 1.74 ± 0.17 | 3 µmol/L | 1.75 ± 0.30 | 0.025 µmol/L | 2.29 ± 0.53 | 3 µmol/L | 1.27 ± 0.13 |
| Crizotinib | 5 µmol/L | 2.06 ± 0.30 | 5 µmol/L | 1.96 ± 0.14 | 0.05 µmol/L | 2.38 ± 0.56 | 4 µmol/L | 1.34 ± 0.15 |
| Crizotinib → | 1 µmol/L | 2.70 ± 0.37 | 1 µmol/L | 1.58 ± 0.24 | 0.025 µmol/L | 2.08 ± 0.49 | 2 µmol/L | 1.74 ± 0.14 |
| Cisplatin | 2 µmol/L | 2.42 ± 0.21 | 2 µmol/L | 0.95 ± 0.03 | 0.05 µmol/L | 1.42 ± 0.06 | 1.89 ± 0.17 |

Cells were treated with the indicated fixed concentration of crizotinib (IC0 and IC50) either simultaneously for 72 h (indicated by “+”), or sequential with 72 h cisplatin preceding 72 h crizotinib or crizotinib preceding cisplatin (indicated by “→”). The simultaneous treatment of LUDLU-1 was performed 2 times, all other conditions were tested at least 3 times. Criz: Crizotinib; CI: Combination index; SEM: Standard error of mean.
In wild no benefit in EGFR mechanisms for this synergy are a decrease in hypoxia and gefitinib. Furthermore, triple combinations of cisplatin, pemetrexed and erlotinib led to synergistic cell death in EGFR mutant NSCLC cell lines and xenografts with erlotinib. Studies showed that platinum analogs in combination with erlotinib treatment was found to be antagonistic in NSCLC cell lines with different genetic backgrounds. Several molecular mechanisms contributed to this synergism. Firstly, pemetrexed increased phosphorylated-EGFR, thus enhancing the effect of EGFR-blocking by erlotinib. Secondly, the combination of both drugs enhanced the reduction of Akt-phosphorylation, leading to increased apoptosis. Finally, the combination of both drugs also decreased the Thymidylate Synthase (TS) in situ activity, which has been correlated with increased pemetrexed sensitivity.

For many combination therapies no appropriate preclinical investigations were performed before starting clinical trials to determine whether synergism could be expected and what would be the most optimal treatment schedule. This also precludes proper patient selection. Possibly, the combination of both EGFR/cMET inhibitors with cisplatin and pemetrexed chemotherapy activates survival mechanisms that abrogate the benefit of inhibiting these receptor tyrosine kinases, although these mechanisms remain to be further investigated.

Other TKIs have been known in vitro to synergize with chemotherapy, such as EGFR-inhibitors with platinum doublet chemotherapy, whereas clinical trials showed no substantial benefit when combining both drugs. Combinations of cisplatin with EGFR-TKIs have been investigated extensively, both in vitro and in vivo. In wild-type EGFR (WT-EGFR) NSCLC cell lines, cisplatin may upregulate phosphorylated EGFR, thus sensitizing these cells to erlotinib; However, in NSCLC cell lines with sensitizing EGFR mutations, combining cisplatin with erlotinib treatment was found to be antagonistic. Other studies showed that platinum analogs in combination with erlotinib led to synergistic cell death in EGFR-mutant NSCLC cell lines and xenografts. Possible mechanisms for this synergy are a decrease in hypoxia-inducible factor 1α (HIF1α), a decrease in c-Myc or cell cycle effects, while also platinum-adduct formation by cisplatin was increased. However, several clinical studies combining cisplatin with EGFR-TKIs showed no benefit in EGFR-WT or in EGFR-mutant patients. Furthermore, triple combinations of cisplatin, pemetrexed and gefitinib; cisplatin, gemcitabine and erlotinib or cisplatin, pemetrexed followed by gefitinib maintenance therapy showed no or only a minor beneficial effect. In contrast, studies investigating the dual combination of erlotinib and pemetrexed, showed synergism in NSCLC cell lines with different genetic backgrounds. Several molecular mechanisms contributed to this synergism. Firstly, pemetrexed increased phosphorylated-EGFR, thus enhancing the effect of EGFR-blocking by erlotinib. Secondly, the combination of both drugs enhanced the reduction of Akt-phosphorylation, leading to increased apoptosis. Finally, the combination of both drugs also decreased the Thymidylate Synthase (TS) in situ activity, which has been correlated with increased pemetrexed sensitivity.

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Given the intertwining of the EGFR and cMET signaling, we opted to test the same combination in EGFR mutant cell lines. These cell lines reflect the NSCLC patient populations with exon 19 deletion, L858R and T790M mutations in EGFR, cMET amplification, and different histological subtypes (adenocarcinoma and squamous cell carcinoma). Despite mimicking several clinical combinations in vitro, the results showed strong antagonism in all the tested treatment schemes.

In conclusion, we show that the combination of the cMET inhibitor crizotinib with cisplatin is moderately to strongly antagonistic in four NSCLC cell lines. This effect was independent of the cMET/EGFR genetic background, the histological subtype of the cells and the used treatment schedule. Our in vitro results suggest an antagonistic effect of combining cMET inhibition with
cisplatin in NSCLC, discouraging further development of this combination in an in vivo and/or clinical setting.

**COMMENTS**

**Background**

During the last decade, several targeted therapies have been developed for the treatment of lung cancer, inhibiting specific receptors in cancer patients. Given the small number of patients eligible for these therapies, cisplatin based therapy still remains the standard of care treatment for most non-small cell lung cancer (NSCLC) patients. The potential benefit of combining cisplatin with targeted therapies, predominantly against the epidermal growth factor receptor (EGFR), has proved to be disappointing. To investigate the potential benefit of combining cisplatin with crizotinib, the authors have performed in vitro studies on a panel of NSCLC lines with different genetic backgrounds.

**Research frontiers**

The combination of a cMET inhibitor and cisplatin has not been investigated in NSCLC patients to date. However, in vitro studies show contradictory results where the outcome is dependent on tumor type and origin. For example, addition of the cMET ligand hepatocyte growth factor (HGF) enhanced cisplatin resistance in seven different NSCLC cell lines.

**Innovations and breakthroughs**

In vitro studies show contradictory results where the outcome is dependent on tumor type and origin. For example, addition of the cMET ligand HGF enhanced cisplatin resistance in seven different NSCLC cell lines. However, another study in SW620 cells, a KRAS mutated colon cancer cell line, showed that conditioned knock-down of cMET did not influence cisplatin sensitivity. In contrast, ovarian cancer cell lines were sensitized towards cisplatin with the addition of HGF. HGF pretreatment of these cells decreased the transcription of protein phosphatase 2A, thus increasing the effect of cisplatin. Here the authors show that the combination of the cMET inhibitor crizotinib with cisplatin is moderately to strongly antagonistic in four NSCLC cell lines. This effect was independent of the cMET/EGFR genetic background, the histological subtype of the cells and the used treatment schedule.

**Applications**

The in vitro results suggest an antagonistic effect of combining cMET inhibition with cisplatin in NSCLC, discouraging further development of this combination in an in vivo and/or clinical setting.

**Terminology**

NSCLC: Non-small cell lung cancer, EGFR: Epidermal growth factor receptor, one of the known drivers of NSCLC.

**Peer-review**

This is an interesting work that will help to understand the molecular mechanism of resistance of EGFR inhibitors and the necessity of continuing search of new investigation for the treatment of such lethal disease that is NSCLC.

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