EGFR- and VEGF(R)-Targeted Small Molecules Show Synergistic Activity in Colorectal Cancer Models Refractory to Combinations of Monoclonal Antibodies

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Abstract

Purpose: Epidermal growth factor receptor (EGFR) and VEGF(R) signaling show extensive cross-talk, providing a rationale for joint targeting of the two pathways. However, combinations of monoclonal antibodies (mAb) targeting EGFR and VEGF showed disappointing activity in patients with colorectal cancer (CRC). We speculated that inhibition of surface receptors and ligands might only partly prevent oncogenic signaling whereas small-molecule tyrosine kinase inhibitors (TKI) would also influence intracellular signaling.

Experimental Design: Mice with CRC xenografts were treated with two TKIs, vargatef and afatinib, or with two mAbs, bevacizumab and cetuximab, and their influence on tumor growth, viability, in vivo DNA synthesis, and the presence of phosphorylated EGFR and VEGFR was determined. The activity of the TKIs was further characterized in CRC cells with different KRAS status.

Results: Vargatef and afatinib together showed strong tumor growth inhibition toward HT-29 xenografts compared with either drug alone, which was associated with a 5-fold increase in apoptotic tumor cell death. In comparison, bevacizumab and cetuximab together were exclusively cytostatic with no more activity than either drug alone. Exposure to the two TKIs was accompanied by a marked decrease of tumor-associated intracellular phospho-VEGFR1 and phospho-EGFR, whereas similar exposure to the two mAbs had no detectable effect. A synergistic activity of vargatef plus afatinib was observed in all eight CRC cell lines examined, independent of KRAS status.

Conclusions: Our results indicate that attenuation of intracellular EGFR and/or VEGF signaling is required for cytotoxic activity. These findings provide a rationale for trials of the TKIs, even in patients with mutant KRAS.

Introduction

The development of anticancer agents targeting oncogenic signaling pathways represents a major conceptual breakthrough. However, in many cases, the clinical outcome has been less than expected, in part, due to the existence of downstream activating mutations, unsuspected feedback loops, and signaling pathway cross-talk. As a result, much effort is currently focused on targeting of several signaling pathways at the same time (1). Cross-talk between the epidermal growth factor receptor (EGFR) and the VEGF signaling pathways plays an important role in tumor growth and survival (2). Activation of EGFR signaling in tumor cells stimulates the production of VEGF, which then acts in a paracrine fashion on surrounding endothelial cells to stimulate their proliferation and migration (2). Several preclinical studies have combined different EGFR- and VEGF(R)-targeted small-molecule tyrosine kinase inhibitors (TKI) or monoclonal antibodies (mAb) with encouraging results (3–8). Bevacizumab, a VEGF-neutralizing mAb, and cetuximab, an EGFR-targeted mAb, are both approved for treatment of colorectal cancer (CRC). Although an early clinical trial (BOND2)
Translational Relevance

Both epidermal growth factor receptor (EGFR)- and VEGF-directed monoclonal antibodies are approved for treatment of colorectal cancer. However, their combination has shown disappointing clinical activity despite the extensive cross-talk between EGFR and VEGF signaling pathways. This article reports that combinations of EGFR- and VEGF-targeted small-molecule tyrosine kinase inhibitors (TKI) inhibit intracellular signaling, trigger apoptotic cell death, and show synergistic antitumor activity in colorectal cancer xenografts and cells, independent of KRAS status. In contrast, combinations of monoclonal antibodies targeting the same pathways were exclusively cytostatic, with no more activity than either drug alone. This work is highly translational and provides a rationale for clinical trials of the TKIs, even in patients with mutant KRAS.

combining bevacizumab and cetuximab looked promising (9), more recent studies (PACCE and CAIRO2) representing almost 1,800 patients showed that the addition of (10), more recent studies (PACCE and CAIRO2) representing almost 1,800 patients showed that the addition of bevacizumab plus chemotherapy alone, was no better than bevacizumab plus chemotherapy alone, even in patients with wild-type KRAS tumors (10, 11). The mechanistic basis for these unexpected results is difficult to establish because no preclinical data are available for the combination of VEGF- and EGFR-targeted mAbs (12), neither with regard to their activity in xenograft models nor with respect to functional biomarkers.

A number of recent findings highlight the importance of intracellular signal transduction in tumor cells. First, it has been shown that the signaling of receptor tyrosine kinases (RTK) such as EGFR and VEGFR is not limited to the RTKs but also inactivated RTKs continue to signal and may even acquire novel functions (13). Second, several studies have shown the presence of internal autocrine (intracrine) VEGF/VEGFR1 signaling in different tumor types (14, 15). This notion is further supported by the observation that deletion of VEGF-A by homologous recombination, and thus extinction of VEGF/VEGFR intracrine signaling, was accompanied by decreased cell growth and increased spontaneous apoptosis of CRC cells (16). Finally, it has been suggested that autocrine VEGF/VEGFR1 signaling synergizes with EGFR to promote tumor cell survival and/or proliferation (17, 18).

An important therapeutic implication of these findings is that strategies to block VEGF or EGFR signaling by inhibition of extracellular ligands or receptors, as is the case for the mAbs, may only prevent part of the oncogenic signaling. In contrast, we would expect that small-molecule TKIs might be able to interfere with internal RTK signaling and cross-talk, including VEGF/VEGFR1 intracrine loop. To test this hypothesis, we selected 2 TKIs including vargatef/BBF 1120, a triple angiokinase inhibitor of VEGFR, PDGFR, and FGFR (19), and afatinib/BIBW 2992, which irreversibly inhibits EGFR and HER2 (20). Vargatef is currently in phase III trials in non–small cell lung cancer (NSCLC) and ovarian cancer, whereas afatinib has reached phase III trials for the treatment of NSCLC and breast cancer.

We now report that vargatef and afatinib together show synergistic activity in CRC models that are refractory to the bevacizumab and cetuximab combination and elucidate the mechanistic differences between the TKIs and the mAbs. In particular, our results show that only TKIs are able to attenuate intracellular EGFR and VEGFR signaling, which is accompanied by the induction of apoptotic cell death. Our findings provide a mechanistic explanation for the failure of the mAbs and indicate that rationally selected EGFR- and VEGF(R)-targeted agents could be combined for clinical benefit.

Materials and Methods

Xenograft models

The antitumor effects of the molecular targeted agents were evaluated in athymic mice (female NMI-Foxn1n, 6 weeks old) from Taconic bearing HT-29 CRC xenografts. Two million cells were injected into the right flank, and the treatments were started when the tumors were palpable (median tumor volume ~100 mm3). The animals were weighed daily and the tumor size was determined 3 times per week. Tumor volumes (mm3) were calculated according to formula: \[(length^2 \times width)/2\]. Boxplot analysis of the weights and tumor volumes was carried out using the GraphPad Prism version 5.00 software (GraphPad Software). Treated/control values were calculated as follows: \[(average\ tumor\ volume\ of\ treated\ animals/average\ tumor\ volume\ of\ control\ animals)\times100\]. Animals were treated according to institutional guidelines.

Immunohistochemistry

All biomarker analyses were carried out with tumors collected after 18-days drug exposure when necrosis is minimal. To measure in vivo DNA synthesis, the thymidine analogue 5-ethyl-2’-deoxyuridine (EdU; Invitrogen) was intraperitoneally administered 48 hours before sacrifice (500 μg). The incorporated EdU was revealed by a fluorescent azide coupling reaction (Click-iT; Invitrogen) of paraffin-embedded tumor samples and counterstained by 4’,6-diamidino-2-phenylindole to reveal the nuclei of individual cells. The proportion of apoptotic tumor cells was scored by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (In Situ Cell Death Detection kit; Roche Applied Science).

The following antibodies were used for immunohistochemistry analysis: anti-phospho-EGFR antibodies (#12351; Santa Cruz), which recognize Tyr1173-phosphorylated EGFR, anti-phospho-VEGFR1 antibodies (#07-758; Millipore), which recognize Tyr1213-phosphorylated VEGFR1, and the relevant Cy3-conjugated secondary antibodies (Jackson Immunoresearch). All images were captured with a fluorescence microscope, and the fluorescence intensities...
were determined by the MetaMorph software (Universal Imaging Corporation) for quantitative analysis.

For the quantitative analysis of the in vivo DNA synthesis (EdU incorporation), the data represent the ratio between EdU-positive cells and the total number of viable cells and are the averages of 5 fields per tumor (each field representing approximately 1,700 cells) from 3 different tumors. For the quantitative determination of apoptosis, the data represent the ratio between TUNEL-positive apoptotic cells and the total area of viable cells and are the averages of 5 fields per tumor for 4 different tumors. For the quantitative analysis of the signal intensity for phospho-EGFR and phospho-VEGFR1, the data represent the average fluorescence intensity of treated tumors, compared with the treatment intensity of control tumors, and are the averages of 5 fields per tumor for 4 different tumors.

**Tumor cells, cytotoxicity assays, and flow cytometric analysis**

Tumor cells were kindly provided by Richard Camalier (Division of Cancer Treatment and Diagnosis Tumor Repository, National Cancer Institute) and by Richard Hamelin (Paris, France). Cellular viability was determined by the MTT viability assay after 120-hour continuous drug exposure as described previously (21). Cell-cycle analysis was carried out as described (22), whereas the proportion of apoptotic cells was characterized by flow cytometry using the APO-BRDU Kit from BD Biosciences. Drug combination effects were determined by the analysis of Chou and Talalay based on the median-effect equation (23, 24) and are indicated in terms of combination index. Data were analyzed by using the concentration effect analysis software (Biosoft). Statistical analysis and graphs were accomplished by GraphPad Prism version 5.00 (GraphPad Software).

**Immunoblot analysis**

Immunoblot analysis was carried out as described previously (25). The primary antibody was directed against p27Kip1 (#3686; Cell Signaling), whereas the secondary antibody was purchased from Jackson ImmunoResearch.

**Results**

**Influence of vargatet, afatinib, and their combination on tumor growth in comparison with bevacizumab and cetuximab**

For these experiments, we used HT-29 xenografts, which is a classical CRC model for the testing of anticancer agents. The animals were exposed to doses that lead to comparable antitumor activity of the 4 agents when given alone. No drug-associated morbidity or mortality was observed for any of the groups.

Simultaneous exposure to bevacizumab or cetuximab was no better than either agent alone (Fig. 1A and C). In contrast, the combination of vargatet and afatinib was associated with a clear synergistic effect compared with either agent alone (Fig. 1B and C).

**Influence of vargatet, afatinib, and their combination on tumor proliferation and viability compared with bevacizumab and cetuximab combinations**

Tumor growth inhibition can be due to cytostatic (cell-cycle related) or cytotoxic effects. To distinguish between these possibilities, we first determined the influence of the 4 agents on in vivo DNA synthesis. Tumor-bearing mice...
were injected with EdU, a thymidine analogue, 48 hours before sacrifice, and the incorporation was determined by a fluorescent-azide coupling reaction. Quantitative image analysis showed that treatment with bevacizumab, cetuximab, or vargatrf alone was accompanied by approximately 40% inhibition of the in vivo DNA synthesis, whereas afatinib inhibited the DNA synthesis by almost 70% (Fig. 2A). Bevacizumab and cetuximab together or vargatrf and afatinib together was no better than the most active of the 2 agents when given alone (Fig. 2A).

Next, the TUNEL assay was used to determine the influence of the 4 agents on apoptotic tumor cell death. Treatment with bevacizumab, cetuximab, or vargatrf alone was not accompanied by any notable increase in apoptosis compared with control tumors, whereas exposure to afatinib doubled the fraction of apoptotic cells (Fig. 2B). Simultaneous exposure to bevacizumab and cetuximab was not accompanied by increased apoptosis, whereas simultaneous exposure to vargatrf and afatinib increased the fraction of apoptotic cells almost 5-fold, compared with vehicle-treated control tumors (Fig. 2B).

Influence of prolonged drug exposure on the phosphorylation of EGFR and VEGFR1

Survival of CRC cells has been linked to internal autocrine (intracrine) signaling. We therefore determined the influence of prolonged drug exposure on receptor autophosphorylation by quantitative immunohistochemistry. HT-29 control tumors displayed a strong signal for phospho-EGFR, which was both membrane-associated and intracellular (Fig. 3A). Exposure to bevacizumab plus cetuximab had modest influence on the intensity (86% ± 7%, compared with vehicle) of phospho-EGFR and no effect on its distribution. In contrast, exposure to vargatrf plus afatinib was accompanied by a pronounced diminution of the total phospho-EGFR signal (36% ± 4%, compared with vehicle) and a reduction of the intracellular fraction (Fig. 3A).

The presence of tumor-associated VEGFR1 has been reported for CRC cells (15, 26) and for tumors in patients with CRC (27). In agreement, control tumors displayed a strong signal for phospho-VEGFR1, which was both membrane-associated and intracellular (Fig. 3B). Exposure to bevacizumab plus cetuximab was accompanied by a decrease of the phospho-VEGFR1 signal to 81% ± 7% of vehicle controls without any detectable alterations of the cellular distribution. Similar exposure to vargatrf plus afatinib was accompanied by a decrease to 52% ± 8% of controls and a marked reduction of the intracellular fraction (Fig. 3B).

Influence of afatinib and vargatrf on the viability of colorectal carcinoma cells

For further characterization, the activity of afatinib toward a CRC cell panel was determined. For comparison, we included 3 reference cell lines expressing high levels of EGFR and/or HER2 including EGFR-overexpressing human epidermoid A431 carcinoma cells, HER2-overexpressing NCI-N87 gastric carcinoma cells, and HER2-overexpressing BT-474 breast carcinoma cells. The results revealed a 130-fold range in the sensitivity to afatinib (Fig. 4A), with IC_{50} values (drug concentration inhibiting cell growth by 50%) ranging from 0.6 to 6.5 μmol/L and an average IC_{50} of 2.2 μmol/L.

Tumor-associated VEGFR1 is believed to influence cellular survival and/or proliferation (14, 15, 18). In agreement, our results show that vargatrf reduced the viability of CRC carcinoma cells, with IC_{50} values ranging from 0.6 to 4.5 μmol/L and an average IC_{50} of 2.2 μmol/L (Fig. 4B).
To determine whether the observed effects were drug-specific or rather reflected the intrinsic sensitivity of the individual cell lines, the IC50 values for vargatef were plotted against the IC50 values for afatinib (Fig. 4C). Data analysis by the Student t test revealed no correlation (r² = 0.14, P = 0.22) between vargatef and afatinib, confirming that the sensitivity to the 2 drugs is mediated by different pathways.

Influence of vargatef, afatinib, and their combination on cell-cycle progression

The growth inhibitory effects of vargatef and afatinib in vitro could be due to cytostatic or cytotoxic effects. Cell-cycle analysis of HT-29 and LS513 cells showed that both vargatef and afatinib induced a pronounced cell-cycle arrest in G1 by 24 hours that lasted throughout the 120-hour incubation period. Interestingly, simultaneous exposure to both drugs was only associated with a marginally increased G1 fraction compared with either agent alone (Fig. 5A and B).

Cell-cycle arrest of erlotinib-treated lung cancer cells has been causally linked to the induction of the cyclin-dependent kinase inhibitor p27Kip1 (28, 29). Our results show that p27Kip1 is also induced in CRC cells exposed to vargatef and/or afatinib (Fig. 5C).

Influence of vargatef, afatinib, and their combination on the viability of colorectal carcinoma cells with different KRAS and BRAF status

Induction of apoptotic cell death was determined by the TUNEL assay. Continued exposure of HT-29 cells to vargatef or afatinib as a single agent was accompanied by induction of apoptotic cell death in at least 10% of the cells after 96-hour drug exposure (Fig. 6A). In contrast, no increased cell death was observed for LS513 cells throughout the 120-hour incubation period (Fig. 6B). Simultaneous exposure to vargatef and afatinib was accompanied by a marked increase of apoptotic HT-29 cells after 72 hours, which reached more than 40% of the total by 120 hours (Fig. 6A). Unexpectedly, simultaneous exposure...
to vargatef and afatinib also induced apoptosis in at least 20% of LS513 cells (Fig. 6B). In confirmation, the analysis of Chou and Talalay of LS513 cells exposed to different concentrations of vargatef and afatinib showed at least additive activity, except at low doses. At drug combinations leading to more than 50% loss of viability, the combination of the 2 drugs was synergistic (Fig. 6C).

To extend these findings, the influence of vargatef and afatinib was determined for a CRC cell panel with different KRAS or BRAF mutational status. The results show that the vargatef and afatinib combination was more cytotoxic than either drug alone for 8 of 8 cell lines tested, regardless of KRAS and BRAF mutational status, or whether the cells displayed the microsatellite instability phenotype (MSI/MIN) or loss of heterozygosity (LOH/CIN/MSS; Fig. 6D).

Discussion

This study was undertaken to determine whether the disappointing results in recent clinical trials with combinations of EGFR- and VEGF-targeted mAbs could be explained by their limited activity on intracellular signaling events. Although several preclinical studies have previously combined different VEGF(R) and EGFR-targeted agents, the present study is, to the best of our knowledge, the only to have compared the activity of TKIs with mAbs in the same in vivo model.

We found that vargatef and afatinib together showed strong tumor growth inhibitory activity toward HT-29 CRC xenografts, compared with either drug alone, which was associated with increased tumor cell death. In comparison, bevacizumab and cetuximab together were no more active than either drug alone and showed exclusively cytostatic activity.

Little is known about how prolonged drug exposure influence RTK autophosphorylation and thus their activity. Only TKIs are likely to inhibit intracellular RTKs following

Figure 4. A, the growth inhibitory activity of afatinib (white columns) or (B) vargatef (dark columns) toward CRC cells was determined by the MTT viability assay after 120 hours of continuous drug exposure and is expressed as IC50 values (drug concentration inhibiting cell growth by 50% compared with untreated controls). The hatched columns correspond to reference cell lines with overexpression of EGFR (A431) or HER2 (NCI-N87, BT-474). All values are averages of at least 3 independent experiments each done in duplicate. C, the IC50 values for vargatef were plotted against the IC50 values for afatinib for each cell line. The results revealed no correlation between the sensitivity to the 2 agents.

Figure 5. The influence of vargatef (）、afatinib (）、or their combination (）on the proportion of (A) HT-29 or (B) LS513 cells in the G1 phase of the cell cycle. Cells were exposed to the IC50 dose of each drug, and the cell-cycle distribution was determined by flow cytometric analysis. All values are averages of 2 independent experiments each done in duplicate. Bars, SD. C, HT-29 cells were exposed to the IC50 dose of each drug, and the protein levels of the cell-cycle regulator p27<sup>KIP1</sup> were determined by immunoblot analysis. The protein levels of β-actin are shown as the loading control. NT, not treated.
short-term exposure. However, because receptor internalization and stability are also influenced by the phosphorylation status, it was possible that long-term exposure to both TKIs and mAbs could modify the levels and cellular distribution of active, phosphorylated RTKs. Control tumors displayed both membrane-associated and intracellular phospho-EGFR and phospho-VEGFR1. Prolonged exposure to bevacizumab plus cetuximab had modest effect on the levels of phospho-EGFR in HT-29 tumors and no detectable influence on the distribution. Similar exposure to vargatef plus afatinib was accompanied by almost 65% reduction of the phospho-EGFR signal and a reduction of the intracellular fraction. Bevacizumab plus cetuximab exposure was accompanied by a 20% diminution of the phospho-VEGFR1 signal and a marked reduction of the intracellular fraction. These results indicate that prolonged exposure to vargatef plus afatinib together reduces the intracellular levels of both phospho-VEGFR1 and phospho-EGFR, whereas similar exposure to bevacizumab and cetuximab combinations has no detectable influence. The close association between attenuation of intracellular phospho-EGFR and phospho-VEGFR1 and the induction of apoptotic cell death in these in vivo models is in line with recent results for cellular models that document the important contribution of intracellular signaling for tumor cell survival (13–18).

Further characterization of vargatef and afatinib in a CRC cell panel revealed that prolonged exposure to both compounds was accompanied by decreased cellular viability. The cytotoxic effects were analyzed according to the Chou and Talalay method. Combination index values of less than 0.8 indicate synergy, values between 0.8 and 1.2 indicate additive effects, and values of more than 1.2 indicate antagonism. D, the influence of vargatef (dark columns), afatinib (white columns), and their combination (gray columns) on the viability of 8 different CRC cell lines was determined by the MTT assay. All values are averages of at least 2 independent experiments each done in duplicate. Bars, SD. mut, mutant; wt, wild-type.
p27Kip1. Vargatef and afatinib together did not result in any further enrichment of G1 phase cells compared with either agent alone, coherent with the in vivo findings. Therefore, one possible explanation for why combinations of EGFR and VEGF(R)-targeted molecules are no better in inhibiting proliferation than the most active of the two when given alone may be that both compounds depend on the activity of the same cell-cycle mediator.

In contrast, vargatef and afatinib together increased the fraction of apoptotic cells, which was particularly striking for LS513 cells, where either drug alone was incapable of inducing apoptosis. Accordingly, Chou and Talalay analysis of LS513 cells exposed to different combinations of vargatef and afatinib showed mostly additive to synergistic effects.

Combinations of vargatef and afatinib were associated with at least additive effects in 8 of 8 CRC models tested, independent of KRAS and BRAF mutational status or the microsatellite instability (MSI/MIN) phenotype. In agreement, it has been reported that the vargatef plus afatinib combination showed activity in mice with Ras-dependent sarcomas (18). Mutation of KRAS or BRAF is a negative predictive factor for EGFR-targeted antibodies in patients with CRC (30), whereas the influence of KRAS mutations is less clear for the TKIs (31). Interestingly, mutant KRAS is also a negative predictive factor for inhibitors of the mTOR and this is observed in both cellular and xenograft models as well as in patients with cancer (32), suggesting that cellular models may be useful for establishing the influence of KRAS status on the response to targeted agents. Therefore, the activity of the vargatef and afatinib combination toward CRC models with mutant KRAS or BRAF is an important observation that merits clinical validation considering that up to 40% of patients with CRC have mutant KRAS whereas approximately 10% have mutant BRAF.

Because both vargatef and afatinib are multi-targeted agents, one could ask to which extend the activity of these compounds depends on the inhibition of VEGF(R) and EGFR. This question has recently been addressed for K5-SOS mice with epidermal carcinomas where keratinocyte-specific deletion of the genes for VEGF and EGFR had comparable influence on tumor growth as pharmacologic inhibition of VEGFR and EGFR signaling by vargatef and afatinib (18). Thus, it seems that inhibition of VEGF(R) and EGFR signaling is an essential contributor to the activity of the vargatef plus afatinib combination. However, it is likely that the additional targets of the 2 drugs also contribute to the antitumor activity. Indeed, system biology models predict that evolvable systems such as RTK networks are resistant to interception of individual components but fragile when subjected to multiple simultaneous perturbations (33), as would be the case for vargatef and afatinib.

Another major question is to which extend the findings presented here are applicable for combinations of other VEGF(R)- and EGFR-directed agents. The biological activity of all TKIs depends on multiple factors including the specificity, the degree, and the duration of target inhibition. Both vargatef and afatinib are multi-targeted agents that are associated with strong RTK inhibition for prolonged periods of time (19, 20). Therefore, although it might be feasible to combine other VEGF(R) and EGFR-targeted agents besides the 2 compounds described here, it is unlikely that all combinations of 2 TKIs, or of 1 TKI with 1 mAb, will be active.

The toxic side effects of combining VEGF(R)- and EGFR-targeted agents are also likely to depend, at least in part, on the properties of the individual compounds. A recent phase I study concluded that continuous daily oral exposure to afatinib was safe and had durable antitumor activity (34) whereas 2 phase I studies reported that continuous vargatef displays a favorable safety and pharmacokinetics profile with first efficacy signals (35, 36). Furthermore, it has been shown that the 2 drugs could be combined in patients with CRC, even if the scheduling and duration remains to be established (37). Monoclonal antibodies directed against growth factor receptors and their ligands principally target the human form of these proteins, for which reason some of the activity of the mAbs may be underestimated in xenograft models. However, we do not believe this is an issue for the present work, because the lack of synergistic activity for the 2 mAb is in line with the clinical findings. Furthermore, this limitation does not apply to our principal finding that elucidates the differential effects of TKI and mAb combinations on intracellular EGFR and VEGF(R) signaling in the tumor cells, which are of human origin.

Taken together, our findings underline the utility of preclinical models and the use of functional biomarkers for the testing of molecular-targeted anticancer agents, alone or in combination. The results elucidate a mechanistic reason for the clinical failure of the 2 mAbs and provide a rationale for trials of the TKIs, even in patients with mutant KRAS.

Disclosure of Potential Conflicts of Interest

The sponsors had no role in the study design, data collection and analysis, interpretation of the results, the preparation of the manuscript, or the decision to submit the manuscript for publication.

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