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"Combination of vandetanib, radiotherapy and irinotecan in the LoVo human colorectal cancer xenograft model"

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Running Title: Vandetanib, radiotherapy and irinotecan in LoVo colorectal cancer

Conflict of Interest: Statement

Anderson Ryan is an employee of AstraZeneca Pharmaceuticals.

Abstract:

Purpose: The tumor growth kinetics of the human LoVo colorectal xenograft model was assessed in response to vandetanib, an orally available receptor tyrosine kinase inhibitor, radiotherapy (RT) or irinotecan (CPT-11), as single therapies and in combination.

Methods and Materials: LoVo cells were injected subcutaneously into the right hind limb (5 x 10^6 cells in 100 μl PBS) of athymic NCR NUM mice and tumors were grown to a volume of 200-300 mm^3 before treatment. Vandetanib was administered at 50 mg/kg daily p.o. for 14 days starting on day 1. RT was given as three fractions (3 x 3 Gy) on days 1, 2 and 3. CPT-

11 was given at 15 mg/kg i.p. on days 1 and 3. Tumor volumes were measured on a daily basis and calculated by measuring tumor diameters with digital calipers in two orthogonal dimensions.

Results: All three single treatments (vandetanib, CPT-11 and radiation) significantly slowed LoVo colorectal tumor growth. Vandetanib significantly increased the antitumor effects of CPT-11 and radiation, when given in combination with either of these treatments. These treatment combinations resulted in a slow tumor growth rate during the two weeks of vandetanib administration. The triple combination of vandetanib, CPT-11, and radiation produced the most marked improvement in response as observed by measurable shrinkage of tumors during the first week of treatment.

Conclusions: The tumor growth delay kinetics observed in this study of the LoVo colorectal model suggest concurrent and sustained post-sequencing of vandetanib with cytotoxic therapy may be beneficial in tumors of this type.

Key Words:

Vandetanib, radiotherapy, CPT-11, LoVo colorectal cancer, angiogenesis inhibitor

Introduction

Colorectal cancer (CRC) remains one of the leading causes of mortality worldwide. In recent years, the most widely used chemotherapy for metastatic CRC, fluoropyrimidine (5-fluorouracil (5-FU)) in combination with folinic acid (FA), has been combined with newer, highly active cytotoxic agents. Among these agents is the DNA topoisomerase I inhibitor, irinotecan (CPT-11) [1], a potent DNA-targeting drug used in patients with CRC that is refractory to treatment with fluorouracil and leucovorin. This cytotoxic agent is, in turn,

currently being combined with new molecular therapies targeting the tumor vasculature and key signaling pathways controlling tumor cell proliferation, angiogenesis and survival in CRC. In this regard, the epidermal growth factor receptor (EGFR) plays an important role in CRC tumor growth and progression [2], and cetuximab, a monoclonal antibody (MAB) specific for EGFR, has been approved for use in combination with CPT-11 in patients with EGFR-expressing CRC refractory to CPT-11-based chemotherapy [3]. In addition, bevacizumab, a MAB specific for vascular endothelial growth factor (VEGF-A), a key player in tumor angiogenesis in CRC as well as other solid tumors, has been approved for the treatment of metastatic CRC in combination with intravenous 5-FU-based chemotherapies [4]. Despite recent improvements in treatment for CRC, a need still remains to improve the performance of existing treatments and to establish the optimum scheduling and dosing of combined therapies.

Vandetanib (ZACTIMA™) is an oral receptor tyrosine kinase inhibitor that, in recombinant enzyme assays, demonstrates potent activity against VEGFR-2 tyrosine kinase (IC_{50} = 40 nmol/L) with additional activity against EGFR (IC_{50} = 500 nmol/L) and RET (IC_{50} = 130 nM) tyrosine kinases [5-8]. Vandetanib currently has orphan-drug status in the USA and Europe for medullary thyroid cancer (MTC) (in which RET activity is important) and is in Phase III development in non-small-cell lung cancer and MTC. Phase II studies are

ongoing to investigate its efficacy in other tumor types, thyroid cancer, hepatocellular carcinoma and glioblastoma.

Vandetanib has been shown to enhance the efficacy of radiotherapy in subcutaneous (s.c.) and orthotopic tumor xenograft models [9-13]. The combination of vandetanib, radiation and current chemotherapeutic agents used in CRC treatment has not been studied to date. Preclinical demonstration of efficacy of a combination protocol with novel agents plus radiation is usually considered crucial prior to clinical evaluation. The purpose of the present study was to examine the effect of vandetanib on the radiation response of a colorectal tumor model when administered in combination with CPT-11. It was hypothesized that simultaneous inhibition of VEGFR and EGFR by vandetanib in combination with the cytotoxic agent CPT-11 would interact to enhance radiation response and tumor control in the human LoVo colorectal tumor cell model. The LoVo colorectal model expresses activated EGFR [14, 15] and is highly vascularized, and therefore is an appropriate model to test the hypothesis.

Materials and Methods

Animal and Tumor Model: LoVo cell suspensions (5 x 10^6 cells in 100 µl phosphate buffered saline) were implanted s.c. into the right hind limbs of 6-8 week-old athymic NCR NUM mice (Taconic Farms, Hudson, NY). A s.c. xenograft model was chosen to facilitate radiation dosing and ease of tumor

measurements. Tumors were allowed to grow for approximately 25 days, until reaching an approximate volume of 200-300 mm^3 at the start of treatment (day 1). All animals were randomized among treatment groups.

Drug Treatment: Vandetanib (AstraZeneca, Macclesfield, UK) was administered by oral gavage (p.o.) at 50 mg/kg daily for 14 days, starting on day 1. Vandetanib dosing in this study was based on previous pharmacokinetic studies in mouse models predicting relevance of this dosing to clinical drug exposure in human patients [16]. CPT-11 was given at 15 mg/kg intraperitoneally (i.p.) on days 1 and 3.

Radiation Treatment: Irradiation was performed on anesthetized mice using X-rays generated by a PanTak, 310 kVe x-ray machine, 0.25 mm Cu + 1 mm Al added filtration, at 125 cGy per min. Dosimetry was performed by an in-the-beam ionization chamber calibrated against a primary standard. Corrections were made daily for humidity, temperature and barometric pressure. Mice were anesthetized with a combination of ketamine and acepromazine at a concentration of 37.5 mg/kg and 0.2 mg/kg, respectively, to provide 25-30 minutes of sedation. Each mouse was confined in a lead casing with its tumor-bearing leg extended through an opening on the side to allow the tumor to be irradiated locally. Radiation was administered as three daily fractions of 3 Gy each on days 1, 2, and 3. On days when radiation was

administered with vandetanib and/or CPT-1, vandetanib and CPT-11 were given approximately 2 hours before radiation, with vandetanib preceding CPT-11 administration.

Tumor Measurement: Tumors were synchronized to be approximately 250 mm^3 at the start of treatment (day 1) and were measured four to five times per week, for up to six weeks of follow-up, or until they reached 2,000 mm^3. Tumor size was determined by direct measurement with calipers and calculated by the formula: (smallest $diameter^2$ x widest diameter)/2. Tumors were not allowed to grow beyond 2,000 mm^3 in accordance with Institutional Animal Care and Use Committee regulations.

In-vivo Tumor Necrosis: Tumors were collected from animals on day 14 following start of treatment for fixation and staining with H&E. The area of necrosis was evaluated by image analysis and expressed as the percentage of the total tumor area.

Statistical Analysis Tumor growth was analyzed via mixed-effects regression, as previously described The method was used because it does not depend on an arbitrary endpoint target tumor size, but utilizes the repeated tumor size measurements obtained over the entire study period, while appropriately handling unbalanced data (i.e., different number of measurements for different animals) and the correlation of each animal's

measurements over time. Mixed-effects regression yields generalizable parameters of interest (e.g., average daily tumor growth rate and tumor doubling time), and can investigate treatment interactions and non-linear patterns of tumor growth. The base-10 logarithm of tumor volume was modeled as a function of time and treatment. Linear or quadratic growth curves over time were fitted to the log-transformed data, depending on growth patterns in each treatment groupAll statistical analyses were conducted in SAS 9.2 (SAS Institute Inc., Cary, NC, 1999-2001).

Results

The experiment involved three different treatments (vandetanib, CPT-11, and radiotherapy), as described above and summarized in Figure 1. Data were collected from a total of 104 animals in eight experimental groups (11-16 animals per group) and are summarized in Figure 2. Starting tumor sizes were comparable across groups, with geometric means ranging from 230 to 257 mm^3 ($p = 0.771$). All treatments were well tolerated in the animals with no observable loss of body weight.

The three single-treatment groups (CPT-11, radiation, or vandetanib), as well as the combination of CPT-11 with radiation (Figure 2) were fitted to log transformed curves, while the three remaining groups that received combination treatments involving vandetanib showed a significantly non-linear tumor growth and were fitted to quadratic curves.

Figure 3 shows the measured geometric mean tumor size graphically over time. Table 1 shows the corresponding calculated tumor growth parameters (daily tumor growth rate and tumor doubling time). Table 2 shows p-values for group comparisons at 7, 14, and 21 days after start of treatment.

The control group had an estimated average daily tumor growth rate of 9.9%, corresponding to an average tumor doubling time of about 7 days (Table 1). All three single treatments resulted in a significant inhibition of tumor growth, compared with the control group (average daily tumor growth rates: CPT-11: 7.1%, $p = 0.015$; radiation: 5.6%, $p = 0.001$; vandetanib: 5.0%, $p = 0.001$) Vandetanib inhibited tumor growth significantly more than CPT-11 ($p = 0.043$) but not radiation ($p = 0.514$); radiation and CPT-11 were not significantly different ($p = 0.139$). The combination of CPT-11 with radiation produced a daily tumor growth rate of 5.1%, which was significantly lower than CPT-11 alone ($p = 0.015$) but comparable to radiation alone ($p = 0.560$). There was no significant (additive) interaction between CPT-11 and radiation ($p = 0.105$).

The remaining three groups which received treatment combinations involving vandetanib (with either CPT-11 or radiation, or with both CPT-11 and radiation), showed significant treatment interactions ($p = 0.001$ for the interaction between vandetanib and CPT-11 and between vandetanib and radiation) and non-linear tumor growth patterns. Compared to single-treatment groups, growth was significantly delayed (and, in the triple-

treatment combination, tumor volume actually decreased) early on, but progressively accelerated later, although it never exceeded that of the untreated controls (Figure 3). Because of the non-linearity of tumor growth in these groups, tumor growth parameters are not constant over time and comparisons depend on the timepoint referenced. Table 2 shows p-values for days 7, 14, and 21.

During the first week of treatment, animals receiving the combination of vandetanib with CPT-11 had average daily tumor growth rate of less than 3.5%, significantly lower than CPT-11 alone and marginally so compared to vandetanib alone ($p = 0.001$ and 0.058, respectively, after 7 days). By the end of the two-week vandetanib treatment, the tumor growth rate in the combination group (4.6%) was still significantly lower than for CPT-11 alone ($p = 0.015$) but comparable to that for vandetanib alone ($p = 0.682$). By the third and fourth weeks, tumor growth had reached levels similar to those seen in the single-treatment groups (Figure 3, Table 1).

The combination of vandetanib with radiation resulted in a similar pattern of non-linear tumor growth inhibition. After the first 7 days, the average daily tumor growth rate of 2.1% was significantly lower than for either radiation alone or vandetanib alone ($p = 0.005$ and 0.019, respectively). After 14 days, the tumor growth rate in the combination group had accelerated to 3.4% and was only marginally lower than for radiation alone and comparable to that for

vandetanib alone (p = 0.080 and 0.212, respectively). By the third and fourth weeks, tumor growth had become similar to that seen in the single-treatment groups (Figure 3, Table 1).

Despite delaying tumor growth in the initial weeks, the treatment combinations induced only modest levels of tumor necrosis (10-20%), with no significant differences between treatment groups (Figure 4).

The pattern of tumor growth in the group that received the triple-treatment combination reflected both the interaction between vandetanib and CPT-11 and that between vandetanib and radiation (as mentioned previously). Thus, during the first week, instead of the delayed tumor growth seen in the two-treatment combinations, tumor volume in the triple-treatment combination actually decreased (p = 0.001 versus vandetanib plus CPT-11, and 0.052 versus vandetanib plus radiation). After that time, similar to the two-treatment combinations that involved vandetanib, tumor growth started accelerating. By the end of the third week, tumor growth in the triple-treatment combination group was similar to that in the two-treatment combination groups involving vandetanib, and by the fourth week, it was similar to that in the single-treatment groups.

Discussion

Relatively little is known about the antitumor effects of combining cytotoxic drugs, radiotherapy, and novel targeted therapies that specifically interfere with signaling pathways controlling cancer proliferation, angiogenesis and survival. In the present study, vandetanib, a potent inhibitor of both VEGFR and EGFR signaling, was combined with CPT-11 and/or radiation, to determine if greater anti-colorectal tumor activity can be obtained.

This study demonstrated that all three single treatments (vandetanib, CPT-11 and radiation) significantly slowed LoVo colorectal tumor growth. Previous studies with single-agent vandetanib demonstrated that chronic oral administration reduced tumor vascularity and tumor growth in a variety of xenograft models, including CRC [7, 17]. In the clinic, the safety and tolerability of vandetanib has been demonstrated in patients with advanced colorectal cancer as well as other solid tumors[18]. Vandetanib induced manageable normal tissue toxicities related to inhibition of EGFR and VEGFR signaling such as diarrhea, rash and hypertension[19, 20]. The effect of combining radiation and vandetanib on normal tissue is currently unknown, however it has been shown in both preclinical and clinical trials that use of VEGF inhibitors with radiation may result in higher rates of normal tissue toxicity such as induction of thrombosis, hemorrhage and bowel toxicities [21-23]. In contrast, it was postulated that combination of radiotherapy with inhibitors of angiogenesis may actually decrease these risks because radiotherapy has been used to prevent hemorrhage [24]. Overall the investigation of agents such

as vandetanib in combination with radiation in normal tissue are lacking, and thus will be a major focus in the future.

As previously discussed, single agent vandetanib has dual tyrosine kinase inhibitory activity against VEGFR-2 and EGFR which allows it to target two key pathways responsible for tumor growth, i.e., tumor angiogenic signaling and tumor cell proliferation. It has been speculated that dual suppression may be critical for sustained suppression of tumor growth, especially since the EGFR and VEGFR pathways are linked and exhibit cross-talk [25]. In addition, vandetanib can also enhance the antiproliferative activity of selective EGFR inhibitors such as cetuximab, thereby potentiating suppression of EGFR signaling[17].

The present study confirmed that vandetanib, chronically administered over two weeks, slowed tumor growth in a colorectal tumor model, and, under the dosing conditions of this study, slowed tumor growth to a greater extent than CPT-11 alone and to a similar level to radiation alone. Moreover, vandetanib significantly increased the antitumor effects of CPT-11 and radiation, when given in combination with either of these treatments. In particular, these treatment combinations resulted in a slow tumor growth rate during the two weeks of vandetanib administration. These results confirm an earlier study by Troiani et al. [26], in which vandetanib (25 mg/kg/day) administered in combination with CPT-11 exhibited high antitumor activity in HT29-tumor-

bearing nude mice. Troiani et al. showed a correlation between this dosing schedule and enhanced EGFR and VEGFR signal inhibition.

In the present study, the triple combination of vandetanib, CPT-11, and radiation produced the most marked improvement in response in the LoVo-tumor-bearing mice. The triple treatment produced a measurable shrinkage of tumors during the first week of treatment. The combination of vandetanib, chemotherapy (gemcitabine), and radiation has also been previously shown to significantly inhibit tumor progression in a pancreatic tumor model [27]. Importantly, the present study also investigated the kinetics of tumor growth, both during and after a course of treatment. It was demonstrated that the addition of vandetanib significantly enhanced the initial antitumor effect of chemo-radiation. However, when vandetanib treatment ended, tumor growth returned to near control (untreated) levels. Therefore, these data support the rationale of adding an anti-vascular agent to cytotoxic therapies and provide valuable information for the design of therapeutic protocols.

The precise mechanisms leading to initial tumor regression with the combined therapies in this study are not known. Analysis of interactions between cytotoxic agents and vandetanib is complex, given that both the tumor cells and the tumor microenvironment are affected. In this connection, radiation can kill not only tumor cells but also endothelial cells of the tumor vasculature, thereby affecting the radiosensitivity of the tumor [28, 29]. In addition, cytotoxic agents have mechanisms of cell killing that are different from the targeted

agent. Both radiation and CPT-11 kill cells through DNA damage. Both chemotherapy and radiation can also alter cellular signaling pathways by inducing EGFR phosphorylation and through the growth factor signaling pathway, contribute to tumor cell proliferation and survival [30-32]. Preclinical studies have also shown that cytotoxic therapy alone, such as radiation, can result in intensification of angiogenic processes [33]. After cytotoxic treatment, up-regulation of vascular growth factors and their receptors occurs which contributes to tumor recurrence and progression [34]. Direct up-regulation of VEGF after irradiation of various cancer cell lines has been reported [35]. Radiation also induces transient tumor hypoxia which results in upregulation of hypoxia inducible factor -1 (HIF-1) which can stimulate VEGF and VEGF-R2 expression. Therefore, simultaneous inhibition of both VEGFR and EGFR signaling through chronic administration of vandetanib in combination with cytotoxic therapy is expected to suppress the upsurge in pro-proliferative and angiogenic signaling resulting from CPT-11 and radiation-induced EGFR and VEGF. This suppression will thereby lead to inhibition of vascular protective mechanisms and growth factor mechanisms contributing to tumor regrowth.

The increased tumor growth that was seen in this study following discontinuation of vandetanib suggests that inhibition of angiogenic and pro-proliferative signaling is readily reversed. The current observations are in agreement with a number of both preclinical and clinical studies showing that tumors can adapt to anti-angiogenic treatment by undergoing “evasive

resistance" to angiogenesis inhibitors [36]. Mechanisms of resistance include upregulation of alternative proangiogenic signaling pathways as well as recruitment of bone marrow-derived proangiogenic cells [37, 38]. In addition, administration of vandetanib itself has been observed to increase VEGF production in certain cancer cell lines as well as in tumor xenografts [39, 40], thereby suggesting an additional contributing mechanism to tumor relapse. More studies will be needed to determine whether additional angiogenic pathways may be induced by triple modality treatment.

Conclusions

The results of this study provide a scientific rationale for testing the combination of vandetanib, CPT-11, and radiation in patients with CRC. Although the best schedule and sequencing for this triple modality treatment has yet to be determined, the tumor growth delay kinetics observed in this study suggest that improvement in colorectal tumor response can be obtained by concurrent and sustained post-sequencing of vandetanib with cytotoxic therapy, keeping in mind that prolonged chronic administration of the receptor tyrosine kinase inhibitors may lead to the development of resistance and the requirement for additional therapeutic agents as seen with other targeted agents, such as imatinib and gefitinib [41, 42].

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Tables

Table 1. Estimates of the average daily tumor growth rate and average tumor doubling time, by treatment group.

Table 2. P-values for comparisons of treatment groups, on days 7, 14 and 21, after the start of treatment.

Figures

Figure 1. Summary of treatment groups.

LoVo cells were implanted s.c. into the right hind limbs of athymic NCR NUM male mice. Mice were randomized into eight experimental groups (11-16 animals per group). Vandetanib was administered at 50 mg/kg daily p.o. for 14 days, starting on day 1. CPT-11 was given at 15 mg/kg i.p. on days 1 and 3. Radiation was given as three fractions (3 x 3 Gy) on days 1, 2, and 3.

Figure 2. Tumor growth curves in LoVo xenografts treated with vandetanib, CPT-11, and/or radiation.

Individual mouse data for eight treatment groups (11-16 animals per group), along with fitted group curves. Vandetanib was administered at 50 mg/kg daily p.o. for 14 days, starting on day 1. CPT-11 was given at 15 mg/kg i.p. on days 1 and 3. Radiation was given as three fractions (3 x 3 Gy) on days 1, 2, and 3.

Figure 3. Estimated geometric mean tumor volume over time in LoVo xenografts treated with vandetanib, CPT-11, and/or radiation.

Vandetanib was administered at 50 mg/kg daily p.o. for 14 days, starting on day 1. CPT-11 was given at 15 mg/kg i.p. on days 1 and 3. Radiation was given as three fractions (3 x 3 Gy) on days 1, 2, and 3.

Figure 4. H&E stained sections of LoVo colorectal xenografts.

All tumors were collected from animals on day 14 following start of treatment. Areas of necrosis are denoted by nec. Magnification 20 X.

(A) Control (untreated) tumor, showing 2% necrosis.

(B) Tumor from animal after administration of last dose of vandetanib, showing 15% necrosis.

(C) Tumor from animal after administration of CPT-11 and RT, showing 20% necrosis.

(D) Tumor from animal after administration of vandetanib and CPT-11, showing 10% necrosis.