Maximum tolerable doses of intravenous zidovudine in combination with 5-fluorouracil and leucovorin in metastatic colorectal cancer patients

Clinical evidence of significant antitumor activity and enhancement of zidovudine-induced DNA single strand breaks in peripheral nuclear blood cells


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Summary

Background: Experimental studies have demonstrated that 5-fluorouracil (5-FU) enhances zidovudine (AZT)-induced DNA strand breaks and cytotoxicity. Phase I studies have demonstrated that the maximum tolerable dose (MTD) of AZT is 8000 mg/m² when administered i.v. over two hours after weekly 5-FU + 1-leucovorin (LV), and that this combination has promising antitumor activity. The purpose of this study was therefore to evaluate the antitumor activity of weekly bolus 5-FU + LV + AZT, and to determine whether 5-FU enhances AZT-induced DNA strand breaks in blood nuclear cells.

Patients and methods: Twenty-nine chemotherapy-naive metastatic colorectal cancer patients with measurable disease entered the study to evaluate the activity of a weekly 5-FU 500 mg/m² i.v. bolus + LV 250 mg/m² i.v. two-hour infusion + AZT 8000 mg/m² i.v. two-hour infusion. In 10 different patients, who during three different weeks received 5-FU + LV, AZT and 5-FU + LV + AZT, DNA strand breaks in blood nuclear cells were determined by a fluorescent analysis of DNA unwinding.

Results: Treatment was generally well tolerated and WHO grades III–IV toxicities, consisting mostly of diarrhea (17%), were uncommon. One patient died of severe diarrhea with consequent hypokalemia and cardiac arrhythmia. All patients were considered evaluable for response, and 3 (10%) complete and 10 (35%) partial responses were observed, for an objective response rate of 45% (95% confidence limit interval 26%–64%). Both 5-FU + LV and AZT decreased the percentage of double stranded DNA in nuclear blood cells. The greatest effect was observed with 5-FU + LV + AZT, which reduced the percentage of double stranded DNA to 50% and 36% after 24 and 48 hours, respectively, and this interaction between 5-FU + LV and AZT was found to be cumulative.

Conclusions: These studies demonstrate that the present dose and schedule of AZT in combination with 5-FU + LV has significant activity in metastatic colorectal cancer and that the combination of 5-FU + LV with AZT increases the amount of DNA damage. Therefore, AZT in combination with 5-FU + LV warrants further study in colorectal cancer.

Key words: AZT, DNA, fluorouracil, phase II, strand breaks, zidovudine

Introduction

Zidovudine (AZT) is a toxic thymidine analogue which was mainly developed as an antiretroviral agent and has shown significant activity in vitro against the human immunodeficiency virus-1 (HIV-1) and clinical utility in patients with AIDS and AIDS-related diseases [1–3]. AZT is phosphorylated intracellularly by thymidine kinase (TK) and can be ultimately incorporated into DNA as AZT triphosphate (AZTTP) where it blocks DNA unwinding. AZT incorporation into DNA, AZT-induced DNA strand breaks and AZT cytotoxicity in cancer cells can be markedly enhanced when AZT is combined with agents that inhibit de novo thymidylate (dTMP) synthesis, such as 5-fluorouracil (5-FU), methotrexate (MTX), hydroxyurea and ZD1694 [5–12]. In fact, these agents, by depleting intracellular thymidine triphosphate (dTTP) pools, facilitate the utilisation of AZTTP in DNA synthesis [7, 8, 13]. We have also demonstrated that this effect is evident also in vivo in nude mice bearing xenografts of the human colorectal cancer cell line HCT-8 [7]. These studies also demonstrated that the enhancement of AZT incorporation into DNA was relatively specific for cancer cells and that this reflects biochemical differences between 'normal' and neoplastic cells with regard to thymidine salvage capacity and transport [7, 14–21].
On the basis of these preclinical observations, we recently conducted a phase I study with intravenous AZT in combination with weekly 5-FU 500 mg/m² and the L-isomer form of leucovorin (LV) 250 mg/m² in chemotherapy-naive metastatic colorectal cancer patients [22]. In this trial, in contrast to previous studies, a short intravenous infusion of AZT was used to reach peak plasma levels similar to those previously found to be effective in experimental murine models [7, 8]. Results demonstrated that AZT doses ≥ 6 g/m² i.v. over 90–120 minutes were able to produce peak plasma concentrations (Cmax) and area under the concentration/time curve (AUC) values similar to those previously demonstrated to be effective in murine tumor models and that the maximum tolerable AZT dose (MTD) was 8 g/m², with hypotension being its dose-limiting toxicity. Furthermore, this combination with 5-FU + LV + AZT demonstrated promising activity, with an objective response rate of 44% (95% confidence interval 27% to 62%) and with a trend toward an increased response rate with higher AZT doses.

The aim of the present phase II study was, therefore, to determine the activity of weekly 5-FU + LV + AZT with AZT given at its MTD of 8 g/m² in previously untreated metastatic colorectal cancer patients. Furthermore, a second objective was to elucidate the biological interaction between 5-FU and AZT in humans and, in particular, to investigate if the enhancement of AZT-induced DNA strand breaks by 5-FU, previously demonstrated in preclinical experimental models [5], could be confirmed also clinically in peripheral blood cells of metastatic colorectal cancer patients.

Patients and methods

Drugs and chemicals

AZT (Retrovir) was generously provided by Glaxo-Wellcome (Beckenham, UK) in 200 mg vials. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Patient selection

Differences in patient accrual were accrued to determine the activity of 5-FU + LV + AZT or the effects on DNA of peripheral nuclear cells. In the phase II study, the main purpose was to determine the activity of AZT at its MTD in combination with 5-FU + LV, the main eligibility criteria included: histologically confirmed diagnosis of colorectal adenocarcinoma with metastatic disease, Eastern Cooperative Oncology Group (ECOG) performance status ≤2, measurable disease, no previous adjuvant or palliative chemotherapy, leukocyte count ≥3500/mm³, platelet count ≥100,000/mm³, serum creatinine ≤1.5 mg/dl, serum bilirubin ≤1.5 mg/dl and transaminases ≤2.5 times normal values. Patients with a history of other malignant tumor (except in situ carcinoma of the cervix or non-melanoma skin cancer), symptomatic cardiac disease or recent history of myocardial infarction or arrhythmia, active infections or cerebral metastases were excluded.

To determine the effects of 5-FU + LV, AZT and 5-FU + LV + AZT on DNA strand breaks of peripheral mononuclear blood cells, the same selection criteria were used, but also patients with non-measurable or non-evaluable metastatic disease could be included in the study.
Analysis of DNA strand breaks

Single strand breaks (SSB) in DNA from peripheral blood nucleated cells were measured before, and 24 and 48 hours after the start of treatment with AZT or 5-FU + LV or 5-FU + LV + AZT. A fluorescent analysis of DNA unwinding, previously described and modified for whole blood cellular populations by collection in heparinized tubes and lysis of erythrocytes without further separation of cells, was used to evaluate the percentage of double-stranded and single-stranded DNA [24]. Briefly, blood samples (10 ml) from patients were collected in heparinized tubes and were mixed with 9 ml of solution A (0.87% NH₄Cl, 10 mM Tris-HCl (pH 7.2)) and held at 0°C for 20-30 min until lysis of erythrocytes was complete. The suspension was centrifuged (0°C, 20 min, 400 g), the pellet suspended in 3 ml of solution A and cells were again centrifuged for 10 min. This pellet was suspended in 2.7 ml of solution B (0.25 M myo-inositol, 10 mM sodium phosphate, 1 mM MgCl₂) to give a total WBC concentration of 5–10 × 10⁶/ml. Aliquots of this suspension (0.2 ml) were distributed into 12 disposable glass tubes designated T, P or B in groups of 4. T tubes were used to estimate the total fluorescence (double-stranded DNA + contaminants), P tubes to estimate the unwinding rate of DNA and B tubes (blank) to estimate the contribution to fluorescence by components other than double-stranded DNA. For this purpose, 0.2 ml of solution C (0.5 M urea, 10 mM NaOH, 2.5 mM cyclenexadimadine tetracateet, and 0.1% sodium deodecsulfate [SDS]) was added to each tube and incubated at 0°C for 10 min. After this time, when cell lysis and chromatim disruption occurred, 0.1 ml of solution D (45% of solution B and 55% 0.2 NaOH) and 0.1 ml of solution E (40% of solution C and 60% of 0.2 NaOH) were gently added to the P and B tubes. After incubation at 0°C for a further 30 min during which the alkali diffused into the lysate to give a final p at of approximately 12.8, the contents of the B tubes were sonicated for 1–2 s to ensure rapid denaturation of the DNA in the alkaline solution; P and B tubes were further incubated at 15°C for 60 min and then denaturation was stopped by chilling to 0°C and adding 0.4 ml of solution F (1 M glucose, and 14 mM β-mercaptoethanol) which lowered the pH to approximately 11.0. The lysates were briefly sonicated to render them homogeneous, diluted with 1.5 ml of solution G (ethidium bromide 6.7 µg/ml, and 133 mM NaOH) and their fluorescence was read at room temperature in a Perkin-Elmer spectrofluorometer (excitation wavelength 520 nm and emission 590 nm). The T tubes differed from the P tubes in that the neutralizing solution E was added before the alkaline solution C and D solutions so that the DNA was never exposed to a denaturing pH. The extent of DNA unwinding after a given time of exposure of cell extracts to alkali was calculated from the fluorescence values of the T, P and B samples. The percent of double stranded DNA is given by the formula (P-B)/(T-B) × 100 where (P-B) provides an estimate of the amount of double-stranded DNA remaining after exposure to alkali for 60 min, and (T-B) an estimate of the amount of double-stranded DNA in the cell extracts. Up to the point at which solution D was added, all steps were carried out under ordinary room illumination; after this, manipulations were carried out in the dark and incubations were in a covered bath. All solutions were kept at 0°C except solutions C and G which were stored at room temperature.

Statistical analysis

In the phase II trial, the optimal stage-two sequential sampling design described by Simon [25] was used to determine the number of patients to be included. Because responses with 5-FU bolus alone are observed in 10%-11% of patients and with 5-FU bolus modulated by LV or MTX in approximately 19%-23% of patients [26, 27], a response rate of 30% or greater with a 5-FU bolus-based regimen should be considered promising. Therefore, the design parameters (α, 0.05; β, 0.20) were chosen to achieve 80% power in this phase II trial. Therefore, the design parameters (α, 0.05; β, 0.20) were chosen to achieve 80% power in this phase II trial. The interaction between 5-FU + LV and AZT on DNA-induced single strand breaks of peripheral nuclear blood cells was analysed using a multiplicative model, as previously described [28, 29]. Briefly, the multiplication of the fraction of double stranded DNA remaining after treatment with 5-FU + LV or AZT (DS-DNA₅-FU+LV or DS-DNA₅-AZT) was compared with the observed result after treatment with 5-FU + LV + AZT. If DS-DNA₅-FU+LV+AZT > (DS-DNA₅-FU+LV × DS-DNA₅-AZT) the interaction is synergistic, if DS-DNA₅-FU+LV+AZT = (DS-DNA₅-FU+LV × DS-DNA₅-AZT) the interaction is cumulative, and if DS-DNA₅-FU+LV+AZT > (DS-DNA₅-FU+LV × DS-DNA₅-AZT) it is antagonistic.

Results

Phase II study

From September 1994 to December 1995, 29 patients entered into this clinical trial. As shown in Table I where the patient characteristics are summarized, the median ECOG PS was 0 (range 0–1), the predominant site of metastasis was the liver and 11 patients had multiple metastatic sites. A total of 585 weekly cycles of 5-FU + LV + AZT were administered with a median of 20 cycles per patient (range 4–34). All patients are evaluable for response after the second accrual stage the treatment was considered promising unless other considerations indicated otherwise.

The Table I. Patients' characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of patients</th>
<th>Male/female</th>
<th>Age</th>
<th>Range</th>
<th>ECOG performance status</th>
<th>Primary</th>
<th>Grading</th>
<th>Sites of disease</th>
<th>LDH (U/L)</th>
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<td>20/9</td>
<td>60</td>
<td>37–74</td>
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<td>Single</td>
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<td>11</td>
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<td>Multiple</td>
<td>365</td>
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| Median                                     | 365             |             |      |             |                          |         |         |                  |           |             |
| Range                                      | 171-980         |             |      |             |                          |         |         |                  |           |             |
toxicity, which consisted of diarrhea, stomatitis, nausea and vomiting, leukopenia, dermatitis, conjunctivitis and hypotension. As shown in Table 2, toxicities were usually mild or moderate (WHO grade I–II); grades III–IV toxicities, consisting of diarrhea (17%), nausea and vomiting (7%), stomatitis (3%), fever (3%) and leukopenia (3%), were uncommon. One patient, who had suffered an episode of myocardial infarction four years before study entry, died of a cardiac arrhythmia which occurred five days after the fourth weekly cycle as a consequence of grade IV diarrhea associated with hypokalemia. Nineteen patients required treatment delays (≥1 week) or 5-FU and AZT dose reductions because of toxicity, with the median 5-FU and AZT dose intensities being, respectively, 442 mg/m²/week (range 280–500) and 6880 mg/m²/week (range 4480–8000). According to the intent to treat analysis, all entered patients were included for evaluation of response. Two additional patients, in whom disease was not actually reevaluated, were considered treatment failures (Table 3); one of these was the patient who died of toxicity after the fourth cycle and the other was one who was lost to follow-up after the fifth cycle and died, presumably of disease progression, four months after treatment start. Overall 3 (10%) complete and 10 (35%) partial responses were observed, for an objective response rate of 45% (95% confidence limit interval 26%–64%). Significant subjective improvement was observed in four of nine patients who initially had disease-related symptoms. The median time to response was two months (range 2 to 4) and the median duration of response eight months (range 2 to 17). Responses occurred in liver (10), lung (3), spleen (1) and retroperitoneum (1). Of the remaining patients 6 (20%) had minor responses, 5 (17%) stable disease and 5 (17%) were treatment failures (three had documented disease progression) (Table 3). The median time to progression and median survival times were 7.5 and 14 months, respectively.

Analysis of DNA strand breaks in peripheral nuclear blood cells

Because previous studies have suggested that 5-FU enhances AZT cytotoxicity by increasing AZT incorporation into DNA and therefore by increasing AZT-induced DNA single-strand breaks [5, 7, 13], 10 patients were studied for the frequency of DNA single-strand breaks in peripheral nuclear blood cells 24 and 48 hours after treatment with AZT 8 g/m², 5-FU 500 mg/m² + LV 250 mg/m² and 5-FU + LV + AZT. All patients had metastatic colorectal cancer (six non-evaluable metastatic disease) and were chemotherapy-naïve. Other characteristics included median age of 61 years, median ECOG PS of 0, nine were males and the most frequent metastatic sites were peritoneum and liver. Treatments were administered in three separate weeks in different sequences that were randomly determined. Results indicate that after treatment with AZT the mean values of double-stranded DNA decreased from approximately 93% to 70% and 59% after 24 and 48 hours, respectively. Similar effects on DNA were observed after treatment with 5-FU + LV, but the greatest effect was observed after treatment with 5-FU + LV + AZT which reduced the percentage of double-stranded DNA to 50% and 36% after 24 and 48 hours, respectively (Figure 1). Seven days after treatment with AZT, 5-FU + LV and 5-FU + LV + AZT, no significant residual DNA damage was observed and the mean values of double-stranded DNA had returned to >90%. The effect of combining 5-FU + LV with AZT on DNA was analysed using the multiplicative model previously described, and it was found to be additive. Of the four patients with evaluable disease one obtained a partial response.

Discussion

Despite the increased accumulation in recent decades of knowledge about the biology and treatment of cancer, the prognosis of metastatic colorectal cancer patients with unresectable disease remains very poor, with a median survival of only 10–12 months, and with fewer than 10% of its victims surviving longer than 36 months [26, 27, 30]. 5-FU has been in use for more than 30 years and is still the drug most often administered. However, 5-FU alone, given as an i.v. bolus, induces responses in only 10%–11% of patients [26, 27]; many attempts have been made to combine it with other agents or to modify the scheduling of its administration in an effort to biochemically modulate its cytotoxicity [31]. Although

| Table 2. Overall worst toxicity (29 evaluable patients). |
|-------------|-----------------|---|---|---|---|
| Adverse event | WHO grade (%) | 1 | 2 | 3 | 4 |
| Nausea/vomiting | 38 | 31 | 7 | 0 | 0 |
| Stomatitis | 34 | 21 | 3 | 0 | 0 |
| Diarrhea | 38 | 27 | 10 | 7 | 0 |
| Dermatitis | 24 | 17 | 0 | 0 | 0 |
| Leukopenia | 41 | 21 | 3 | 0 | 0 |
| Hypotension | 14 | 0 | 0 | 0 | 0 |
| Fever | 17 | 21 | 3 | 0 | 0 |
| Conjunctivitis | 38 | 24 | 0 | 0 | 0 |
| Alopecia | 10 | 7 | 0 | 0 | 0 |

| Table 3. Objective responses. |
|-----------------------------|-----------------|
| Response (WHO) | No. pts (%) |
| Complete response | 3 (10%) |
| Partial response | 10 (35%) |
| Complete + partial | 13 (45%) |
| Minor response | 6 (21%) |
| Stable disease | 5 (17%) |
| Treatment failure | 5 (17%) |
| Total | 29 (100%) |
clinical improvement has been obtained by combining 5-FU with LV or MTX or by administering 5-FU in protracted continuous infusion, survival has been only marginally improved and results remain unsatisfactory [26, 27, 32, 33]. More recently, new studies [34-41] have reported promising results from the use of high doses of 5-FU given as a 24-48-hour continuous infusion, or by combining a 5-FU continuous infusion with a 5-FU bolus + MTX, or by administering 5-FU as a circadian modulated continuous infusion, or with new agents such as tomudex, irinotecan, doxifluridine and oxaliplatin, and further study is warranted.

Recent experimental and clinical studies have suggested that a new approach to improving the treatment of colorectal cancer might be to combine a toxic thymidine analogue, such as AZT, with an inhibitor of dTMP synthesis such as 5-FU, MTX, tomudex and others [5-12]. Indeed, these agents facilitate the incorporation of AZTTP into DNA by lowering intracellular dTTP pools and thus enhancing DNA damage and cytotoxicity [7, 8, 13]. Interestingly, experimental studies have demonstrated that this effect is more evident in colorectal cancer cells because of an elevated thymidine kinase activity (which activates AZT to AZTTP) and the lack of an efficient concentrative nucleoside transport for thymidine, since thymidine competes with AZT for phosphorylation and incorporation into DNA [7, 14-21]. In initial phase I studies [42, 43] AZT, in combination with 5-FU and LV, was administered orally or in protracted (48 hours) intravenous infusions that did not render possible attainment of plasma AZT concentrations that had been demonstrated to be effective in nude mice bearing tumour xenografts (0.5-1 mmol/L) [7, 8]. Subsequent phase I studies, therefore, have used shorter intravenous infusions of high doses of AZT which have allowed achievement of peak plasma concentrations similar to those previously obtained in preclinical animal models and rendered possible a promising activity in previously untreated metastatic colorectal cancer patients [22, 44, 45]. However, these studies did not demonstrate an activity for 5-FU + LV + AZT in heavily pretreated metastatic colorectal cancer patients resistant to 5-FU [42, 43, 45]. This is not surprising, not only because heavily pretreated patients with bulky disease and chemoresistant tumours very rarely respond to any further chemotherapy, but also because the mechanism of action of the 5-FU + AZT combination requires that 5-FU possess some degree of activity. Indeed, if thymidylate synthase (TS) is not inhibited and therefore dTTP pools are not lowered, AZT incorporation into DNA is negligible and does not lead to a detectable clinical activity [13]. Of interest is the fact that these phase I studies [42, 43] demonstrated a dose-dependent biologic effect of AZT manifested by an increase in DNA strand breaks in peripheral nuclear blood cells. However, the possible enhancement by 5-FU of AZT incorporation into DNA and therefore of AZT-induced DNA single strand breaks, was not investigated.

In the present study, we evaluated the activity of a combination of 5-FU bolus + LV with high doses of intravenous AZT, administered at its MTD as a short (120') intravenous infusion, in chemotherapy-naive metastatic colorectal cancer patients, and we also investigated whether the enhancement of AZT-induced DNA strand breaks by 5-FU, previously demonstrated in experimental models, could be confirmed clinically in peripheral nuclear blood cells. Leucovorin was added to 5-FU to enhance TS inhibition, and therefore the depletion of dTTP pools, and AZT was administered starting 60' after 5-FU to ensure that high AZT tumour concentrations were present when TS was inhibited [46]. Although the response rate observed in this trial has a wide 95% confidence limit interval (26%-64%), the antitumor activity of this new combination appears promising; in fact, it compares favourably with that of similar regimens with weekly bolus 5-FU plus LV without AZT (response rates 20%-25%) [26], and it is in the same range as that of more demanding regimens in which 24-48-hour or protracted infusions of 5-FU or chronomodulated infusions of 5-FU plus oxaliplatin have been used [34-37]. These results, therefore, confirm the elevated activity of 5-FU + LV + AZT already
observed in the previous phase I studies in chemother-
apy-naive patients [22, 45]. This study also demonstrates
that the combination of 5-FU + LV with AZT increases
the amount of DNA damage and this might reflect an
increased incorporation of AZT into DNA; neverthe-
less, because 5-FU + LV alone also induced DNA strand
breaks, other mechanisms could be involved. Interest-
ingly, the effect of combining 5-FU + LV with AZT on
DNA was only additive and not synergistic as previously
observed in colorectal cancer cells in experimental mod-
els [5]. A possible explanation is that blood cells, despite
having an elevated TK activity, also possess an active
thymidine transport that could partially protect them
from the cytotoxic effects of AZT due to the competition
between thymidine and AZT, [7, 18, 19]. Therefore, it will
be important to study the induction of DNA damage in
cancer cells of colorectal cancer patients after treatment
with 5-FU + LV and AZT to determine whether these
effects on DNA are similar to those observed in blood
cells or, as demonstrated in experimental models, are
more pronounced and the interaction between 5-FU +
LV and AZT is synergistic.

In conclusion, because of these and previous findings
with 5-FU + LV + AZT in metastatic colorectal cancer,
further studies are warranted. In particular, these results
will require confirmation in randomized trials in which
the effects of this new combination on survival will also
be determined. Furthermore, because of recent experi-
mental findings demonstrating that AZT might circum-
vent cisplatinum resistance and synergistically poten-
tiate the cytotoxicity of cisplatinum + 5-FU [47], and
also that alfa-interferon enhances AZT cytotoxicity [48],
phase I–II studies clinically testing these observations
should be performed. Some have already been initiated
[49–51]. Most notably, a phase I study in 12 patients with
metastatic gastrointestinal carcinomas demonstrates the
feasibility of a combination with 5-FU + LV + AZT and
cisplatin, and some activity also in chemotherapy-pre-
treated patients [51]; furthermore, a phase II study in
HTLV-I associated adult T-cell leukemia-lymphoma
with a combination of AZT and alfa-interferon demon-
strates a significant activity for this combination, with
eight of nine patients obtaining an objective response
[49]. Finally, it will be of interest to combine AZT with
5-FU administered in continuous infusion because of its
more specific effect on TS when compared with 5-FU
bolus [52]; similarly, it will be interesting to combine
AZT with the new folate-based TS inhibitors such as
tomudex which, being very specific and effective inhibi-
tors of TS, might enhance AZT antitumor activity even
more efficiently than 5-FU.

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