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The synergistic interaction of gemcitabine and cytosine arabinoside with the ribonucleotide reductase inhibitor triapine is schedule dependent

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ABSTRACT

Gemcitabine and ara-C have multiple mechanisms of action: DNA incorporation and for gemcitabine also ribonucleotide reductase (RNR) inhibition. Since dCTP competes with their incorporation into DNA, dCTP depletion can potentiate their cytotoxicity. We investigated whether additional RNR inhibition by Triapine (3-AP), a new potent RNR inhibitor, enhanced cytotoxicity of gemcitabine and ara-C in four non-small-cell-lung-cancer (NSCLC) cell lines, using the multiple-drug-effect analysis.

Simultaneous and sequential exposure (preexposure to 3-AP for 24 h) in a constant molar ratio of 3-AP and gemcitabine was antagonistic/additive in all cell lines. Preexposure to 3-AP at an IC_{25} concentration for 24 h before variable concentrations of gemcitabine was synergistic. RNR inhibition by 3-AP resulted in a more synergistic interaction in combination with ara-C, which does not inhibit RNR.

Two cell lines with pronounced synergism (SW1573) or antagonism (H460) for gemcitabine/3-AP, were evaluated for accumulation of the active metabolites, dFdCTP and ara-CTP. Simultaneous exposure induced no or a small increase, but ara-CTP increased after pretreatment with 3-AP, 4-fold in SW1573 cells, but not in H460 (<1.5 fold). Ara-C and gemcitabine incorporation into DNA were more pronounced (about 2-fold increased) for sequential treatment in SW1573 compared to H460 cells (<1.5 fold). This was not related to the activity and expression of deoxycytidine kinase and the M2 subunit of RNR.

In conclusion, RNR inhibition by 3-AP prior to gemcitabine or ara-C exposure stimulates accumulation of the active metabolites and incorporation into DNA. The combination 3-AP/Ara-C is more synergistic than 3-AP/gemcitabine possibly because gemcitabine already inhibits RNR, but ara-C does not.

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Abbreviations: dFdC, gemcitabine (2′,2′-difluorodeoxycytidine; Gemzar); Ara-C, cytosine arabinoside; dCK, deoxycytidine kinase; dFdCDP, gemcitabine diphosphate; dFdCTP, gemcitabine triphosphate; dCTP, deoxycytidine triphosphate; RNR, ribonucleotide reductase; dNTP, deoxynucleoside triphosphate; dCDA, deoxycytidine deaminase; dCMPD, deoxycytidylate deaminase; HU, hydroxyurea; HCT, a-(N)-heterocyclic carboxaldehyde thiosemicarbazone; Fe, iron; 5-HP, 5-hydroxypyridine-2-carboxaldehyde thiosemicarbazone; 3-AP; Triapine, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; TCA, trichloroacetic acid; SRB, sulforhodamine-B; CI, Combination Index; dATP, deoxyadenosine triphosphate; dGTP, deoxyguanosine triphosphate; dTTP, deoxythymidine triphosphate

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1. Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC, Gemzar) is a deoxynucleoside analogue used in the treatment of non-small-cell-lung-cancer (NSCLC) [1]. It is frequently combined with other anti-tumor agents such as taxanes and platinum based analogues [1]. Gemcitabine is transported into the cell by nucleoside transporters where it is phosphorylated to its monophosphate by deoxycytidine kinase (dCK) and subsequently to its active metabolites gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP) [2] (Fig. 1). The main mechanism of action of gemcitabine is incorporation into DNA, for which it competes with deoxycytidine triphosphate (dCTP) [3]. dFdCDP inhibits ribonucleotide reductase (RNR) [4], which is increased in several in vitro [2] and in vivo [5] models of acquired resistance to gemcitabine (Fig. 1). RNR is responsible for catalyzing the reduction of ribonucleotides to their corresponding deoxyribonucleotides essential for DNA synthesis and repair of DNA damage. Inhibition of RNR leads to decreased cellular deoxynucleoside triphosphate (dNTP) pools, including dCTP [6]. The latter effect leads to self-potentiation because dCTP competes with dFdCTP for incorporation into DNA [7]. In addition, since dCTP is a feedback inhibitor of dCK, a decrease in dCTP pools will stimulate gemcitabine phosphorylation. Gemcitabine can be inactivated by deoxycytidine deaminase (dCDA) and deoxycytidylate deaminase (dCMPD) [7].

Cytosine arabinoside (Ara-C) is a deoxynucleoside analogue which has no activity against solid tumors, but is frequently used in the treatment of hematological malignancies [8]. The initial activation of Ara-C is also mediated by dCK, while its active metabolite ara-CTP is also incorporated into DNA [9], leading to inhibition of DNA polymerase (Fig. 1). Similar to gemcitabine, ara-C is also transported into the cell by nucleoside transporters and can also be inactivated by dCDA and dCMPD. In contrast to gemcitabine, ara-C is not able to inhibit RNR (Fig. 1).

RNR is a tetramer consisting of two non-identical homodimers. The two identical M2 subunits regulate the substrate specificity of the enzyme, while the other two identical M1 subunits are responsible for the activity by binding the ribonucleotides and allosteric effectors [10,11]. Two types of RNR exist with two different M2 homologues; the p53 independent form (hRRM2) that is linked with the cell cycle and growth control mechanisms and the recently identified p53 dependent RNR (p53R2) that forms a complex with p53 and is thought to be involved in DNA repair in both proliferating and resting cells [12–14]. P53 can interact with p53R2 and hRRM2 at the protein level to regulate RNR activity [15]. The classic RNR inhibitor hydroxyurea (HU) is a poor inhibitor of RNR and has moderate anticancer activity [16]. Other types of RNR inhibitors include deoxynucleoside analogues, polyhydroxy-substituted benzohydroxamic acid derivatives such as amidox, didox and trimidox [17] and iron chelators such as the thiocarbazones. These chelators target the iron in the M2 subunit of the RNR enzyme. 5-Hydroxypyridine-2-carboxaldehyde thiosemicarbazone (5-HP) is 1000-fold more potent than HU, but clinical results were disappointing because it was rapidly glucuronidated and excreted [18,19]. Its analogue 3-Aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, Triapine) is resistant to glucuronidation, while HU resistant cancer cells are still sensitive to 3-AP [20,21]. Initial in vitro and in vivo anti-tumor activity were promising [20,22] and the compound is currently being evaluated in several phase I and

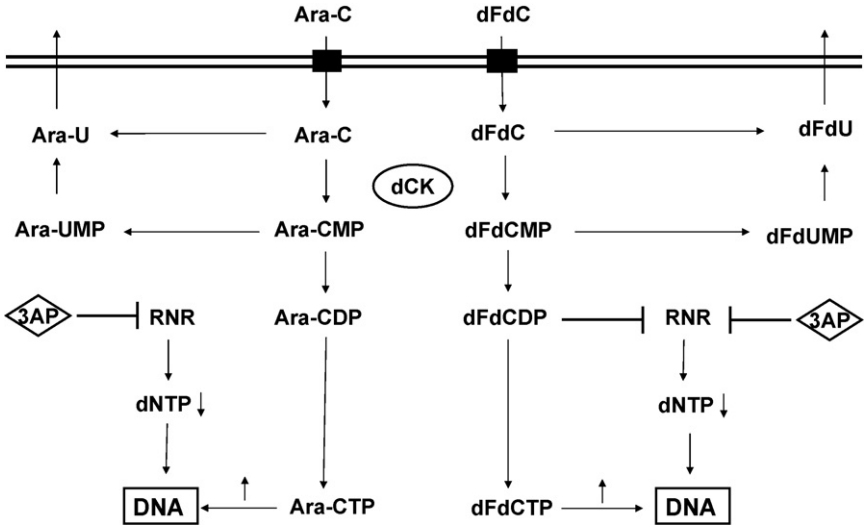

Fig. 1 – Metabolism and hypothetical interaction of deoxynucleoside analogues ara-C (left) or gemcitabine (right) with the RNR inhibitor 3-AP. Both ara-C and gemcitabine are transported into the cell by nucleoside analogue transporters and are phosphorylated by deoxycytidine kinase (dCK) to their monophosphates (ara-CMP, dFdCMP) and subsequently to its diphosphate (ara-CDP dFdCDP) and triphosphate (ara-CTP dFdCTP). Gemcitabine and ara-C triphosphates are incorporated into the DNA leading to DNA damage. Gemcitabine can reduce dNTP pools, but ara-C not. The ribonucleotide reductase (RNR) inhibitor 3-AP will deplete dNTP pools. Decrease of dCTP levels will lead to enhanced ara-C and gemcitabine incorporation into the DNA, since the feedback inhibition by dCTP is diminished. The gemcitabine metabolite dFdCDP already inhibits RNR and can cause some dCTP depletion, but less then for 3-AP. Ara-C does not affect RNR, therefore the interaction between 3-AP and ara-C might to be more synergistic than with gemcitabine.

II trials. 3-AP was well tolerated in patients at daily infusions for 5 days [23]. A prolonged 96 h continuous infusion was more promising [24] and showed some activity in metastatic breast cancer patients. 3-AP was synergistic with DNA damaging agents such as etoposide, cisplatin and doxorubicin in L1210 bearing mice [20], but data on solid tumor cell lines are not available.

The aim of this study was to investigate whether additional RNR inhibition by 3-AP would enhance cytotoxicity of gemcitabine in NSCLC cells using different administration schedules. In order to investigate how much RNR inhibition is involved in this interaction we compared the combination of 3-AP and gemcitabine with the combination of 3-AP and ara-C, which does not inhibit RNR itself. We hypothesized that additional RNR inhibition by the combination of 3-AP and gemcitabine is synergistic, while the combination of ara-C and 3-AP would be more synergistic, resulting in enhanced accumulation of the respective triphosphates and incorporation of gemcitabine and ara-C into DNA.

2. Materials and methods

2.1. Drugs and chemicals

Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, with L-Glutamine and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) were obtained from BioWhittaker (Verviers, Belgium). Both (non-dialyzed) fetal calf serum (FCS) and Hank's balanced salt solution (HBSS) were purchased from Gibco Europe (Paisly, UK). Cytosine arabinoside (ara-C) was from Sigma (St. Louis, MO, USA), gemcitabine (2′,2′-difluorodeoxycytidine, dFdC, Gemzar) was provided by Eli Lilly Research Laboratories, (Indianapolis, IN, USA) and triapine (3-AP) was from Vion Pharmaceuticals (New Haven, CT, USA). 5-^{3}H-gemcitabine (11 Ci/mmol) and 5-^{3}H-AraC (27 Ci/mmol) were purchased from Moravek Biochemicals.

All other chemicals were of analytical grade and commercially available, unless otherwise specified.

2.2. Cell culture

Four non-small-cell-lung-cancer cell lines were used in this study; A549, H292, H460 and SW1573. SW1573 cells were cultured in DMEM medium containing 10% FCS and 20 mM HEPES. The other NSCLC cell lines were cultured in RPMI medium containing 10% FCS and 20 mM HEPES. Cells were maintained at 37 °C under an atmosphere of 5% CO_2.

2.3. Characterization of cell lines

The cell lines were characterized on proteins involved in the gemcitabine and Ara-C metabolism. The RNR protein expression was determined by using Western blotting as described in ref. [5] with minimal modifications. The antibody directed to the M1 subunit of RNR2 was from Chemicon and that for the M2 subunit of RNR from Santa Cruz Biotechnologies. The dCK activity was determined by using radioactive assays as earlier described in Kroep et al. [25] with [^{3}H]-CdA as the substrate. CdA is a very specific dCK substrate and not a substrate for other deoxynucleoside kinases, e.g. TK2 [26].

2.4. Growth inhibition studies

Growth inhibitory effects of gemcitabine, ara-C and 3-AP were evaluated with the SRB assay. NSCLC cells (5000 cells/well) seeded in 96-wells flat-bottomed Greiner plates were exposed to various drug concentrations for 72 h whereafter the SRB assay as performed as described in ref. [27].

Drug interactions were studied by using the multiple drug effect analysis of Chou and Talalay [28] (Calcusyn software, BioSoft, Ferguson, MO, 1996) which enables to determine antagonistic, additive or synergistic interactions. These different kinds of interaction are given by the Combination Index (CI) which is calculated using the formula: $CI = [(D)_1/(D_{1-FA})_1] + [(D)_2/(D_{1-FA})_2] + [\alpha(D)_1\ (D)_2/(D_{1-FA})_1\ (D_{1-FA})_2]$. The parameters $(D)_1$ and $(D)_2$ represent the doses of the combination of drugs in a fixed ratio, whereas $(D_{1-FA})_1$ and $(D_{1-FA})_2$ are the doses of the individual drugs, resulting in the effect 1−FA (Fraction Affected) and $\alpha = 1$ for mutually non-exclusive drugs. The CI of 0.1–0.3 represents strong synergism, CI: 0.3–0.7: synergism, CI: 0.7–0.85: moderate synergism, CI: 0.85–0.90: slight synergism, CI: 0.9–1.1: additive, CI: 1.2–1.45: moderate antagonism, CI: 1.45–3.3: antagonism. In the CI–FA plot the CI values at FA > 0.5 are evaluated and per experiment only the CI values at FA 0.5, 0.75 and 0.9 are used to calculate a mean CI value per experiment. From the mediated values a mean between the experiments was calculated.

2.5. dFdCTP and AraCTP accumulation

The accumulation of dFdCTP and ara-CTP was measured by high performance liquid chromatography (HPLC). SW1573 and H460 cells were seeded at a cell density of 1×10^6 cells/well and 0.5×10^6 cells/well, respectively. Two different schedules of administration were used. Controls were incubated for similar time periods with either ara-C or gemcitabine, without 3-AP. For the simultaneous exposure, cells were exposed to the IC_{25} concentration of 3-AP and 1 μM gemcitabine or 10 μM AraC for 4 or 24 h at 37 °C. For the sequential exposure, cells were first exposed to the IC_{25} concentration of 3-AP for 24 h at 37 °C, whereafter 1 μM gemcitabine or 10 μM ara-C was added and incubated for an additional 4 or 24 h at 37 °C. Retention of the drugs was also measured, by removal of the medium, followed by culture in drug free medium for 4 h. After harvesting the cells were extracted with TCA, and nucleotides were analyzed by HPLC [29].

2.6. DNA incorporation

To determine the gemcitabine and ara-C incorporation into the DNA, tritiated gemcitabine and ara-C were used as earlier described in Bergman et al. [30]. SW1573 and H460 cells were seeded in 96 wells MultiScreen-GV Millipore plates in a volume of 90 μl at a cell density of 1×10^5cells/well. For the sequential exposure, 100 μl of 3-AP resulting in the final IC_{25} concentration was immediately added. Cells were incubated for 24 h at 37 °C, whereafter 10 μl of the hot/cold mix (0.23 μCi) of gemcitabine or ara-C was added resulting in an final

concentration of 1 or 10 μM, respectively. For the simultaneous exposure, 100 μl of 3-AP resulting in the final IC_{25} concentration was added immediately followed by 10 μl of the hot/cold mix (0.23 μCi) of gemcitabine or ara-C. After addition of the radioactive gemcitabine or ara-C, cells were incubated for 24 h at 37 °C. Subsequently, medium was removed by suction through the filters. After washing steps with 8% TCA and PBS, cells were incubated at 37 °C with or without 0.2 μg RNAse A in PBS to differentiate between DNA and RNA incorporation of gemcitabine. The filters were removed after washing steps with cold 8% TCA, H_2O and 70% ethanol. Shaking in 2 M NaOH enabled solubilization of the DNA and RNA. Finally, 5 ml of counting liquid was added and radioactivity was counted for 5 min.

2.7. Statistics

In order to evaluate the effect of 3-AP on ara-CTP and dFdCTP accumulation and on their incorporation into DNA we used the Student's *t*-test for paired samples. Data were also evaluated with the paired Mann–Whitney *U*-test.

3. Results

3.1. Characterization of cell lines

A549, H292, H460 and SW1573 have been used extensively for mechanistic studies of the activities of drugs commonly used in NSCLC and were therefore included in this study. As shown in Fig. 2, the cell lines showed no relevant differences in protein expression of RNR M2 subunit, but that for RNR M1 subunit was about 2-fold lower in SW1573 cells, while dCK activities differed also 2-fold, being lowest in H460 cells and highest in H292 cells (Fig. 3).

3.2. Growth inhibition studies

No differences in sensitivity to 3-AP or gemcitabine were observed in the NSCLC cell lines (Table 1). However, ara-C sensitivities differed considerably. H460 was the most sensitive cell line to ara-C, while SW1573 was highly resistant.

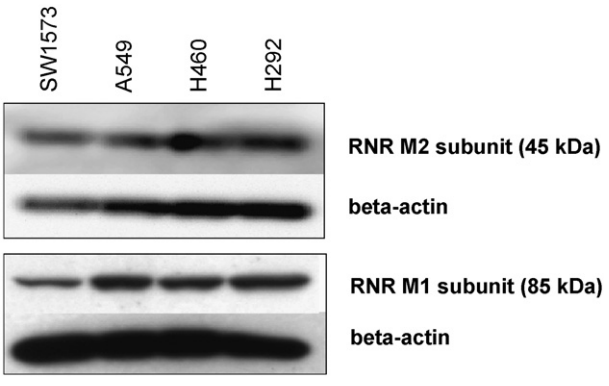

Fig. 2 – RNR M1 and M2 subunit protein expression. Similar amounts of protein were loaded onto the gel: when corrected for β-actin loading only SW1573 cell showed a lower RNR M1 expression.

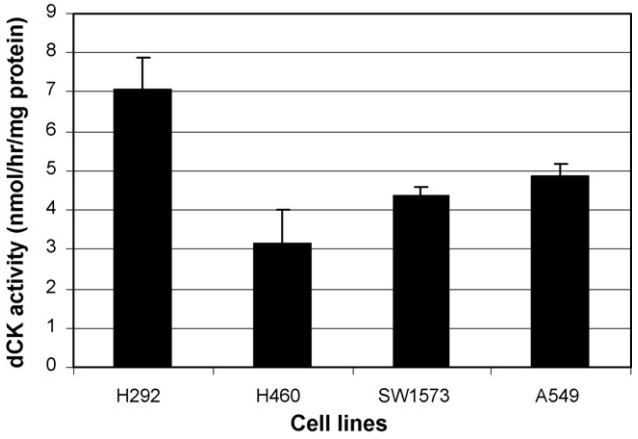

Fig. 3 – dCK activity in NSCLC cell lines. dCK activity expressed per mg protein per hour at 50 μM CdA. Values are means ± S.E.M. of at least three separate experiments.

Initial interactions were studied at a fixed (Table 2) ratio based on the IC_{50} concentrations of the drugs (concentration ratio of 1:200 for gemcitabine and 3-AP, respectively). Both simultaneous exposure and sequential (second drug added after 24 h) exposure showed slight synergistic, additive and moderate antagonistic interactions. Since 3-AP might not exert sufficient RNR inhibition at the lower concentrations and might need some time to exert sufficient RNR inhibition, we subsequently investigated whether the growth is more effectively inhibited when 3-AP was added at an IC_{25} concentration, 24 h prior to addition of variable concentrations of gemcitabine. The IC25 concentration is high enough to exert considerable RNR inhibition [20]. For this schedule, stronger synergistic interactions were found (Table 2, Fig. 4). Using these conditions 3-AP showed considerable synergism in three of the four cell lines with CI values varying from 0.35 to 0.69 (Table 2). Only in H460 a slight antagonism was observed.

Since gemcitabine inhibits RNR by itself, we wondered whether potentiation of a deoxynucleoside analogue without activity against RNR would be more pronounced. For this purpose we used ara-C. 3-AP interaction with ara-C was much more synergistic than the interaction of gemcitabine with 3-AP, with a CI value of 0.2 in SW1573, H292 and A549 cells. In the ara-C sensitive H460 cells, synergism was less pronounced, but still a CI of 0.7 was found.

Table 1 – Sensitivity of NSCLC cell lines to gemcitabine, 3-AP and ara-C

	Gemcitabine	3-AP	Ara-C
H292	5.3 ± 0.3	1012 ± 95	780 ± 214
H460	4.9 ± 0.1	979 ± 34	173 ± 39
SW1573	4.0 ± 0.4	1040 ± 19	10500 ± 866
A549	4.5 ± 0.4	1160 ± 163	620 ± 116

Sensitivity to the drugs (as IC_{50} in nM) was determined using the SRB assay with 72-h exposure. Values are means ± S.E.M. of at least three separate experiments.

Table 2 – Interaction of 3-AP with gemcitabine and Ara-C

Combination Index (CI)	H292	H460	SW1573	A549
Sim: dFdC + 3-AP (fixed ratio 1:200)	1.37 ± 0.28	0.99 ± 0.18	1.25 ± 0.10	1.62 ± 0.35
Seq: 3-AP → dFdC (fixed ratio 1:200)	2.03 ± 0.34	1.43 ± 0.37	1.51 ± 0.26	0.83 ± 0.13
Seq: 3-AP → dFdC variable ratio, 3-AP constant	0.69 ± 0.09	1.21 ± 0.14	0.56 ± 0.05	0.35 ± 0.02
Seq: 3-AP → AraC variable ratio, 3-AP constant	0.23 ± 0.04	0.77 ± 0.04	0.21 ± 0.08	0.21 ± 0.05

Combination indices for simultaneous (sim) and sequential (seq) combinations of gemcitabine/Ara-C with 3-AP in NSCLC cell lines. The ratio 1:200 refers to the constant ratio of drug concentration (IC50:IC50 concentration of gemcitabine and 3-AP). Cells were also exposed to an IC_{25} concentration of 3-AP (3-AP constant) 24 h prior to addition of variable concentrations gemcitabine (dFdC variable) or Ara-C (AraC variable). For evaluation of possible synergism, the median drug effect analysis method of Chou and Talalay (Biosoft) was used. In the CI–FA plot, the CI values at FA > 0.5 were evaluated and per experiment only the CI values at FA 0.5, 0.75 and 0.9 were mediated. Values are means ± S.E.M. of at least three separate experiments.

3.3. *dFdCTP and AraCTP accumulation*

In order to investigate the mechanism of the interaction we selected two cell lines with pronounced synergism and antagonism of the combination of 3-AP and gemcitabine, SW1573 and H460, respectively. In these cell lines dFdCTP and Ara-CTP accumulation were evaluated.

As illustrated in Fig. 5, the accumulation of dFdCTP was higher in SW1573 compared to H460 cells, both at 4 and 24 h exposure. Simultaneous exposure for 4 h marginally increased dFdCTP accumulation, but pretreatment with 3-AP increased accumulation about 2-fold in both SW1573 and H460 cells. SW1573 cells accumulated more dFdCTP which was also retained longer than in H460 cells. In SW1573 cells, 24 h simultaneous exposure hardly increased the dFdCTP accumulation, but pretreatment with 3-AP resulted in a 1.5-fold increase. Despite lack of synergism, pretreatment with 3-AP increased dFdCTP pools almost 2-fold in H460 cells. However, retention of dFdCTP pools was shorter in H460 cells compared to SW1573.

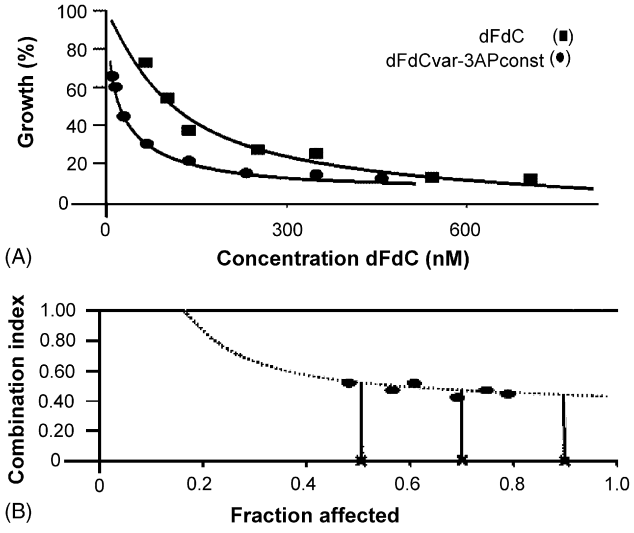

Fig. 4 – (A) Growth inhibition of SW1573 cells after 3-AP pretreatment (IC25) with variable concentrations of gemcitabine. (B) CI–FA plot of the same experiment that represents the calculated interaction between both drugs. The CI values of the FA > 0.5 (at FA 0.5, 0.75 and 0.9) are used to calculate an average CI value of 0.5 indicating a synergistic interaction.

Ara-CTP accumulation was much lower in both cell lines compared to dFdCTP (Fig. 6). However, 3-AP stimulated ara-CTP accumulation to a much larger extent, more than 2-fold in SW1573 at simultaneous exposure and about 4-fold at 3-AP pretreatment. In H460 cells, in which synergism was less, the effects were also lower. The retention of ara-CTP was less than that of dFdCTP, but pretreatment with 3AP increased the retention of ara-CTP in both cell lines.

3.4. *DNA incorporation*

The incorporation of gemcitabine and ara-C into DNA in sequential treatment was higher when compared with simultaneous treatment for SW1573 and H460 cells (Fig. 7), especially for ara-C. The higher synergistic interaction of 3-AP and gemcitabine in SW1573 was related to a 2-fold higher incorporation of gemcitabine into DNA compared to H460. In both cell lines, less gemcitabine was incorporated than ara-C into DNA (SW1573; 3.2 versus 4.1 and H460 2.9 versus 5.6 pmol/24 h, respectively). No incorporation of gemcitabine into RNA was observed in SW1573 and H460 cells (data not shown). In SW1573 cells, the simultaneous and sequential exposure to gemcitabine and 3-AP resulted in an 1.5- and 2.5-fold increased gemcitabine incorporation, respectively, while in H460 cells both simultaneous and sequential exposure resulted in 1.5-fold increased gemcitabine incorporation into DNA. For ara-C, incorporation into DNA after simultaneous exposure was lower compared to sequential exposure. Simultaneous exposure did not result in an increase at all when compared with the single drug exposure.

4. Discussion

In this paper, we demonstrate a synergistic interaction between gemcitabine and ara-C with 3-AP. Pretreatment with 3-AP followed by gemcitabine or ara-C was more effective than simultaneous exposure. Synergism was related to increased accumulation of the triphosphates dFdCTP and ara-CTP, respectively, and increased incorporation of both gemcitabine and ara-C into DNA. It has been shown earlier that the extent of DNA incorporation of both drugs is related to more cell kill [31].

In line with moderate differences in dCK activity, no differences in gemcitabine sensitivity were found. These small differences in dCK activities might be compensated by other

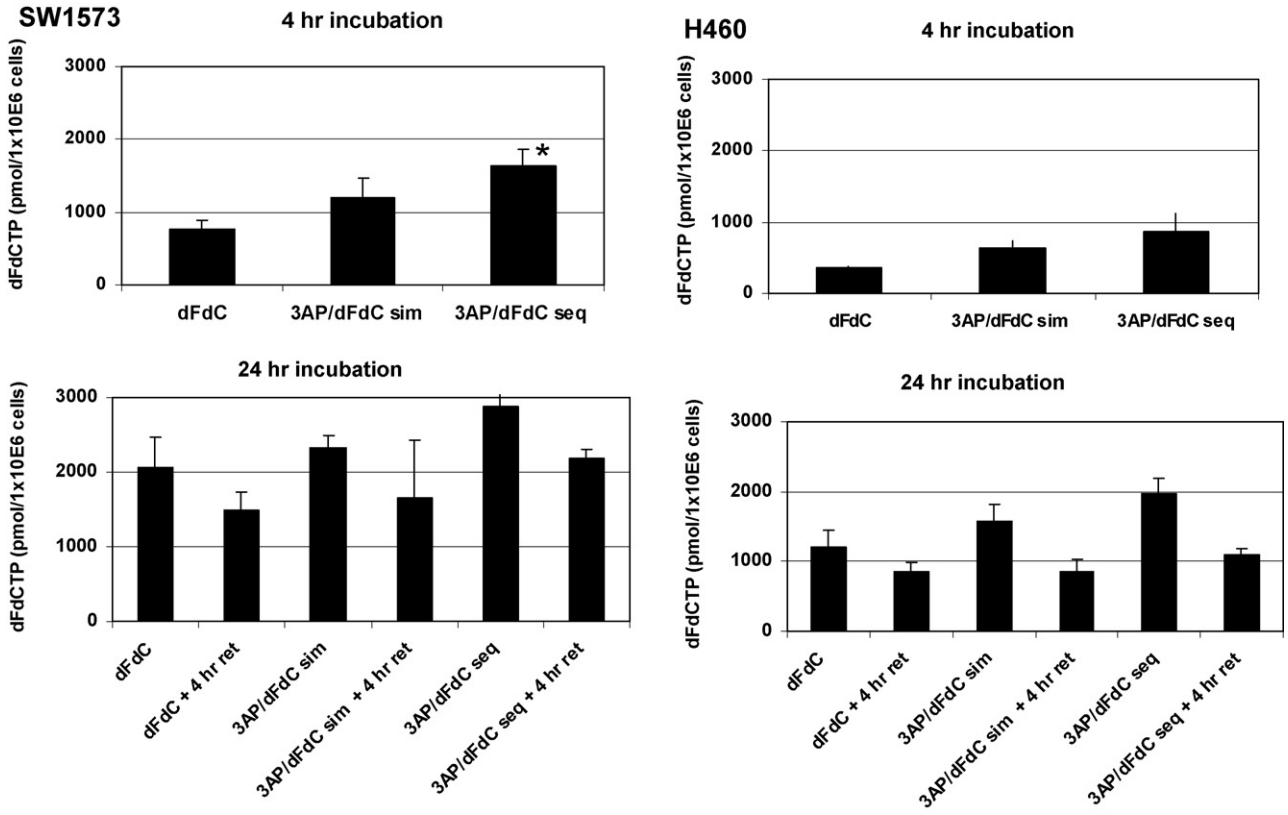

Fig. 5 – Upper panel: dFdCTP accumulation in SW1573 (left) and H460 (right) after 4 h exposure to gemcitabine (dFdC), simultaneous exposure to gemcitabine and 3-AP (3-AP/dFdC sim) and sequential exposure to 24 h pretreatment with 3-AP followed by 4 h gemcitabine (3-AP/dFdC seq). Lower panel: dFdCTP accumulation in SW1573 (left) and H460 (right) after 24 h exposure to gemcitabine (dFdC), 4 h retention after 24 h exposure to gemcitabine (dFdC 4 h ret), exposure to 3-AP and gemcitabine simultaneous (3-AP/dFdC sim), 4 h retention after 24 h exposure to 3-AP and gemcitabine simultaneous (3-AP/dFdC sim 4 h ret), sequential exposure to 24 h pretreatment with 3-AP followed by 24 h gemcitabine (3-AP/dFdC seq) and 4 h retention after sequential exposure to 24 h pretreatment with 3-AP followed by 24 h gemcitabine (3-AP/dFdC seq 4 h ret). 3-AP was used at an IC_{25} concentration and concentration of gemcitabine used was 1 μM. Retention of dFdCTP was measured after 4 h incubation in drug free medium. Values are means ± S.E.M. of at least three separate experiments. A paired Student's t-test was used to evaluate whether the effect of 3-AP was significant. Compared to gemcitabine alone (4 or 24 h) the effect was significant at: $^{*}P < 0.05$ or $^{**}P < 0.01$. A Mann–Whitney test gave similar results.

mechanisms of resistance, e.g. nucleoside transport or deoxycytidine deaminase activity, resulting in equal sensitivities (Table 2). The moderate differences in RNR expression are less likely to explain the gemcitabine sensitivity, and are in line with sensitivity to 3-AP between the cell lines. Sensitivity to ara-C differed considerably between the cell lines. The relative resistance of SW1573 to ara-C cannot be explained by its dCK activity. Other mechanisms of resistance might play a role. Moreover, substrate specific dCK activity has been previously described [32].

In all cell lines tested, synergism was found between gemcitabine and ara-C with 3-AP. Gemcitabine is known to inhibit RNR [4] and gemcitabine resistance has been associated with increased RNR expression [5]. Inhibition of RNR by gemcitabine leads to a partial depletion of dNTP pools most pronounced for dATP and dGTP, but less for dCTP, while dTTP pools increased [6]. When these dNTPs are depleted, gemcitabine might upregulate the transcription factor E2F-1 which plays a pivotal role in cell-cycle control [33]. An E2F-1 induced S-phase entry [34,35] might increase gemcitabine incorporation into DNA. At higher gemcitabine concentrations a depletion of dCTP pools was also observed [6], stimulating DNA incorporation of gemcitabine by decreased competition, resulting in increased DNA damage and apoptosis. Moreover, a decrease in dCTP pools results in decreased feedback inhibition of dCK and increased phosphorylation of gemcitabine [7].

Because gemcitabine only moderately affects the dCTP pools, an additional effect of a potent RNR inhibitor was expected. Additional RNR inhibition by 3-AP resulted in marked synergism with gemcitabine, which was related to higher dFdCTP pools, possibly as a result of decreased feedback inhibition of dCK, and increased incorporation into DNA, due to decreased competition with dCTP for DNA polymerase. Our results are in agreement with a study which showed enhanced chemosensitivity to gemcitabine in pancreatic adenocarcinoma cells in combination with RNA interference, targeting the R2 subunit of RNR [36]. In vivo synergy of the combination of 3-AP with DNA damaging agents such as etoposide, cisplatin and doxorubicin was previously found in L1210 murine leukemia bearing mice [20],

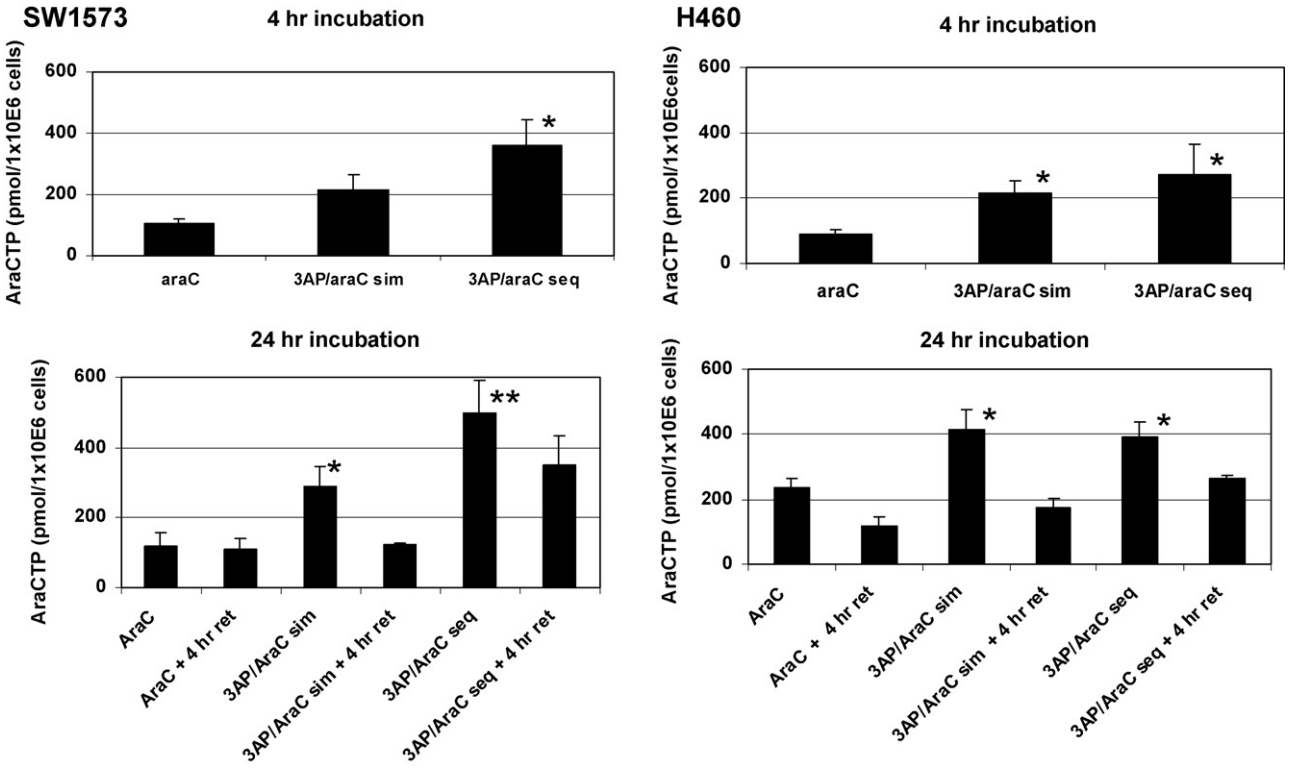

Fig. 6 – Upper panel: ara-CTP accumulation in SW1573 (left) and H460 (right) after 4 h exposure to ara-C (ara-C), simultaneous exposure to ara-C and 3-AP (3-AP/ara-C sim) and sequential exposure to 24 h pretreatment with 3-AP followed by 4 h ara-C (3-AP/ara-C seq). Lower panel: ara-CTP accumulation in SW1573 (left) and H460 (right) after 24 h exposure to ara-C (ara-C), 4 h retention after 24 h exposure to ara-C (ara-C 4 h ret), exposure to 3-AP and ara-C simultaneous (3-AP/ara-C sim), 4 h retention after 24 h exposure to 3-AP and ara-C simultaneous (3-AP/ara-C sim 4 h ret), sequential exposure to 24 h pretreatment with 3-AP followed by 24 h ara-C (3-AP/ara-C seq) and 4 h retention after sequential exposure to 24 h pretreatment with 3-AP followed by 24 h ara-C (3-AP/ara-C seq 4 h ret). 3-AP was used at an IC_{25} concentration and concentration of Ara-C used was 10 μM. Retention of ara-CTP was measured after 4 h incubation in drug free medium. Values are means ± S.E.M. of at least three separate experiments. A paired Student's *t*-test was used to evaluate whether the effect of 3-AP was significant. Compared to gemcitabine alone (4 or 24 h) the effect was significant at: $^{*}P < 0.05$ or $^{**}P < 0.01$. A Mann–Whitney test gave similar results.

but data on gemcitabine and ara-C in solid tumor cell lines are not available.

Since ara-C does not inhibit RNR activity, the use of ara-C in these experiments enabled us to evaluate the role of RNR in the mechanism of action of gemcitabine. The absence of RNR inhibition by ara-C was expected to result in higher synergism of the combination of 3-AP with ara-C, compared to the combination with gemcitabine. Indeed, the combination of ara-C and 3-AP was more synergistic than the combination with gemcitabine, which was related to a higher relative increase in ara-CTP pools. Also in 10 separate acute myeloid leukemia patient samples the combination of ara-C and the RNR inhibitors gemcitabine and cladribine showed marked synergism [37]. These results suggest that inhibition of RNR activity by gemcitabine plays an important role in gemcitabine's growth inhibiting properties. In addition to the direct effects of 3-AP on triphosphate accumulation, we also observed a longer retention of the triphosphates. Normally, dFdCTP shows a biphasic elimination profile and ara-CTP a monophasic much faster elimination [2]. A 4-h measurement of ara-CTP and dFdCTP gave a good insight in the difference between ara-CTP and dFdCTP retention (longer for dFdCTP), but 3-AP pretreatment clearly prolonged the retention of ara-CTP and to a lesser extent that of dFdCTP. Apparently, the imbalance in dNTP pools and decrease in dCTP play a role in the retention as well.

However, the RNR inhibiting properties of gemcitabine and 3-AP differ in binding sites in the multisubunit enzyme. Gemcitabine targets the M1 subunit that binds the ribonucleotides and allosteric effectors, while 3-AP binds to the M2 subunit that contains a tyrosyl radical stabilized by Fe (catalytic site). 3-AP inhibits the catalytic activity of the wild type (RRM2) and the p53 dependent (p53R2) RNR forms with almost equal efficiency that supports the high potential of RNR inhibition [38], resulting in depletion of all dNTPs.

The synergistic interaction of the combination of the RNR inhibitor 3-AP with ara-C and gemcitabine is schedule dependent, with a better effect of pretreatment with 3-AP. Apparently, decreased dNTP pools before treatment with the deoxynucleoside analogues facilitate the formation of ara-CTP and dFdCTP and their incorporation into DNA. Possibly, a decrease of the dCTP level is the major determinant of this

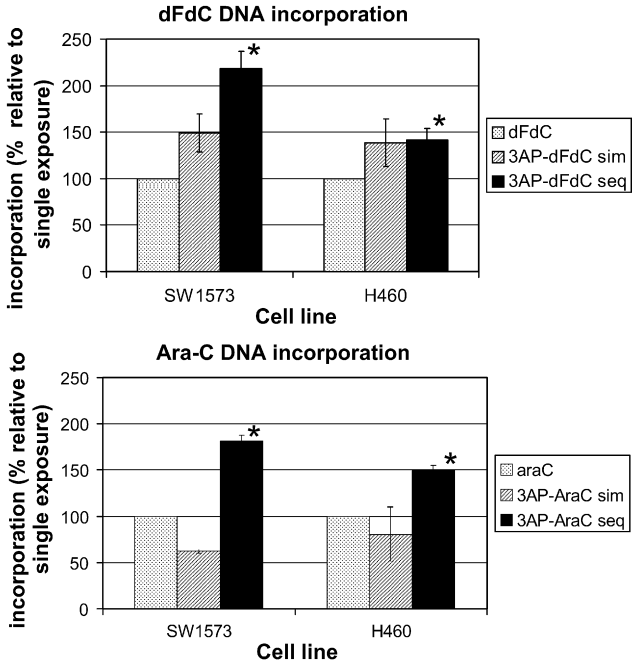

Fig. 7 – Relative gemcitabine (dFdC) and ara-C incorporation into the DNA of SW1573 and H460 cells after 24 h exposure to 1 μM gemcitabine or 10 μM ara-C, respectively. Cells were exposed to 3-AP at IC_{25} concentrations within each separate experiment. Single drug exposure is set on 100% and means ± S.E.M. were calculated from the percentages of each experiment (at three separate experiments). Upper panel: gemcitabine incorporation into DNA after gemcitabine, gemcitabine and 3-AP simultaneous (3AP-dFdC sim) and 24 h preincubation with 3-AP followed by gemcitabine (3AP-dFdC seq). Lower panel: ara-C incorporation into DNA after ara-C, ara-C and 3-AP simultaneous (3AP-AraC sim) and 24 h preincubation with 3-AP followed by ara-C (3AP-AraC seq). In SW1573 cells gemcitabine and ara-C incorporation into DNA was 3.2 and 4.1 pmol/24 h after single drug exposure and in H460 cells 2.9 and 5.6 pmol/24 h respectively. A paired Student's *t*-test was used to evaluate whether the effect of 3-AP was significant. Compared to gemcitabine alone (4 or 24 h) the effect was significant at: $^{*}P < 0.05$ or $^{**}P < 0.01$.

effect. Preincubation with the less potent RNR inhibitor trimidox demonstrated that a significant decrease in dCTP pools synergistically increased the inhibition of colony formation by ara-C in HL60 leukemia cells [39]. Also pretreatment of human CEM leukemia cells with the RNR inhibiting hydroxyisoindole dione derivatives ISID and MISID followed by ara-C resulted in synergistic interaction which was related to increased ara-CTP pools and increased DNA fragmentation [40]. Finch et al. did not show an increased survival of mice bearing L1210 leukemia treated with the combination of 3-AP with gemcitabine or ara-C [20]. However, lack of potentiation might be related to the used schedule of administration which was possibly not optimal.

The synergistic interaction between gemcitabine or ara-C and 3-AP is related to increased levels of the respective triphosphate pools and increased incorporation of the drugs into DNA. However, other mechanisms of synergy between gemcitabine and ara-C should also be considered. The drugs might interact at the level of interference with cell-cycle progression and apoptosis mediating proteins. For example, progression from the G0/1 to S-phase was attenuated by RNR inhibition, which might be enforced in combination with gemcitabine which has the same effect on cell-cycle regulation [41,42]. Human leukemia cells died by apoptosis upon RNR inhibition, which was related to an upregulation of the receptor mediated caspases-8 and the mitochondria dependent caspase-9 [43]. Since gemcitabine also induces apoptosis, however, exclusively via caspase-8, the drugs might synergize by supplementing the different apoptotic pathways [44].

The combination of gemcitabine and ara-C in combination with 3-AP is currently being evaluated in clinical trials. In a Phase I trial, 3-AP was well tolerated in patients at daily infusions for 5 days [45]. Using a prolonged 96 h continuous infusion [24,46] activity was shown as a single agent in metastatic breast cancer and advanced leukemia patients. A Phase I study with the combination of gemcitabine and 3-AP in solid tumors [47] and two Phase I studies with the combination of ara-C and 3-AP in hematological cancers have been completed. Yee et al. showed activity in acute leukemia or myelodysplastic syndrome patients using a 6 h 3-AP infusion followed immediately by an 18-h infusion ara-C for 5 consecutive days [48]. Phase II studies of the gemcitabine and 3-AP combination in biliary cancer, pancreatic cancer and NSCLC have been initiated.

Taken together, the combination of the RNR inhibitor 3-AP with gemcitabine or ara-C is schedule dependent and is most effective when 3-AP is administered prior to the deoxynucleoside analogues. These studies might be of value to develop new effective combination chemotherapy regimens for treatment of cancer.

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