Antiproliferative effects of sapacitabine (CYC682), a novel 2′-deoxyctydine-derivative, in human cancer cells

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This study assessed the antiproliferative activity of sapacitabine (CYC682, CS-682) in a panel of 10 human cancer cell lines with varying degrees of resistance or sensitivity to the commonly used nucleoside analogues ara-C and gemcitabine. Growth inhibition studies using sapacitabine and CNDAC were performed in the panel of cell lines and compared with both nucleoside analogues and other anticancer compounds including oxaliplatin, doxorubicin, docetaxel and seliciclib. Sapacitabine displayed antiproliferative activity across a range of concentrations in a variety of cell lines, including those shown to be resistant to several anticancer drugs. Sapacitabine is biotransformed by plasma, gut and liver amidases into CNDAC and causes cell cycle arrest predominantly in the G2/M phase. No clear correlation was observed between sensitivity to sapacitabine and the expression of critical factors involved in resistance to nucleoside analogues such as deoxycytidine kinase (dCK), human equilibrative nucleoside transporter 1, cytosolic 5′-nucleotidase and DNA polymerase-α. However, sapacitabine showed cytotoxic activity against dCK-deficient L1210 cells indicating that in some cells, a dCK-independent mechanism of action may be involved. In addition, sapacitabine showed a synergistic effect when combined with gemcitabine and sequence-specific synergy with doxorubicin and oxaliplatin. Sapacitabine is therefore a good candidate for further evaluation in combination with currently used anticancer agents in tumour types with unmet needs.

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Sapacitabine (Figure 1A; CYC682, Cyclacel Ltd, Dundee, UK; CS-682, Sankyo, Tokyo, Japan) is a novel 2′-deoxyctydine analogue (1-(2-C-cyano-2-deoxy-β-D-arabino-pentafuranosyl)N4-palmitoylcytosine), which was synthesised by incorporating an additional N4-palmitoyl group to protect the amino group (Kaneko et al, 1997). The rational of adding a N4-palmitoyl moiety to CNDAC was performed in the panel of cell lines and compared with both nucleoside analogues and other anticancer compounds including oxaliplatin, doxorubicin, docetaxel and seliciclib. Sapacitabine displayed antiproliferative activity across a range of concentrations in a variety of cell lines, including those shown to be resistant to several anticancer drugs. Sapacitabine is biotransformed by plasma, gut and liver amidases into CNDAC and causes cell cycle arrest predominantly in the G2/M phase. No clear correlation was observed between sensitivity to sapacitabine and the expression of critical factors involved in resistance to nucleoside analogues such as deoxycytidine kinase (dCK), human equilibrative nucleoside transporter 1, cytosolic 5′-nucleotidase and DNA polymerase-α. However, sapacitabine showed cytotoxic activity against dCK-deficient L1210 cells indicating that in some cells, a dCK-independent mechanism of action may be involved. In addition, sapacitabine showed a synergistic effect when combined with gemcitabine and sequence-specific synergy with doxorubicin and oxaliplatin. Sapacitabine is therefore a good candidate for further evaluation in combination with currently used anticancer agents in tumour types with unmet needs.

Although they have a similar mechanism of action, preliminary in vitro and in vivo investigations have shown that CNDAC and sapacitabine may display overlapping cytotoxic effects with some cancer cells being more sensitive to sapacitabine than CNDAC. Furthermore, data have suggested that the sapacitabine cytotoxicity profile may differ from that of other nucleoside analogues (Kaneko et al, 1997; Faivre et al, 1999; Hanaoka et al, 1999; Zhang et al, 1999; Wu et al, 2003; Matsuda and Sasaki, 2004). Therefore, further investigations are required to ascertain the exact mechanism of action of sapacitabine, to evaluate the toxicity of combinations with other compounds and to screen the range of its activity in various tumour systems that may eventually lead to clinical application.

In this study, we tested the antiproliferative activity and cell cycle effects of sapacitabine against a panel of colon, breast, lung and ovarian cancer cell lines considered as being representative of...
tumours warranting the development of this novel anticancer agent. The antiproliferative effects of sapacitabine were observed in a variety of cell lines resistant to other anticancer drugs. Levels of mRNA expression of critical factors including various transporters and enzymes involved in resistance to nucleoside analogues, such as dCK, human equilibrative nucleoside transporter 1 (hENT1), cytosolic 5'-nucleotidase (cN-II) and DNA polymerase-α (POL), were determined in all cell lines and correlated with sapacitabine cytotoxicity. Sapacitabine also showed cytotoxic activity against dCK-deficient L1210 cells.

Considering that this compound may be amenable to clinical trials in combination with other anticancer agents, combinations of sapacitabine with several anticancer agents commonly used in practice, such as gemcitabine, oxaliplatin, doxorubicin, docetaxel and seliciclib, a novel CDK inhibitor (McClue et al, 2002; Meijer and Raymond, 2003), were also explored.

**MATERIALS AND METHODS**

**Cell lines**

A panel of colon (HT29, HCT116, COLO205, HCC2998), breast (MCF7, MDA-MB-435), lung (HOP62, HOP92), ovarian (OVCAR3, IGROV1) cancer cell lines was purchased from the ATCC (Rockville, MD, USA). Cells were grown as monolayers in RPMI
medium supplemented with 10% fetal calf serum (Invitrogen, Cergy-Pontoise, France), 2 mM glutamine, 100 units ml⁻¹ penicillin and 100 µM ml⁻¹ streptomycin.

Cell cytotoxicity assays

All the data generated were the result of three separate experiments performed in duplicate. Cell viability was determined using the MTT assay, which was carried out as described previously (Hansen et al, 1989). Briefly, cells were seeded in 96-well plates at a density of 2 × 10⁴ cells per well. Cells were incubated for 120 h and then 0.4 mg ml⁻¹ of MTT dye (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; Sigma, Saint-Quentin Fallavier, France) was added for 4 h at 37 °C. The monolayer was suspended in 0.1 ml of DMSO and the absorbance at 560 nm was measured using a microplate reader (Dynatech, Ann Arbor, MI, USA). Positive and negative controls included wells with untreated cells or medium containing MTT with no cells, respectively. The conversion of yellow water-soluble tetrazolium MTT into purple insoluble formazan is catalysed by mitochondrial dehydrogenases and is used to estimate the number of viable cells. The control value corresponding to untreated cells was taken as 100% and the viability of treated samples was expressed as a percentage of the control. IC₅₀ values were determined as concentrations that reduced cell viability by 50%.

For single agent studies, cells were seeded and allowed to settle for 24 h before treatment with increasing concentrations of sapacitabine, CNDAC, seliciclib (CycC202, R-roscovitine; Meijer and Raymond, 2003), a novel cyclin dependent kinase inhibitor, doxorubicin, docetaxel, cisplatin, oxaliplatin, ara-C, 5-FU or gemcitabine for 48 h. After incubation, the cells were allowed to recover in compound-free medium for 72 h, before determination of growth inhibition using the MTT assay.

For sequential or simultaneous studies, three schedules were implemented comprising sequential exposure to sapacitabine (48 h) followed by the second agent (24 h); sequential exposure with one anticancer agent (24 h) followed by treatment with sapacitabine (for 48 h); and simultaneous exposure to both agents (48 h). In sequential exposure schedules, cells were seeded and allowed to grow in the presence of various concentrations of sapacitabine, doxorubicin, docetaxel, seliciclib, oxaliplatin or gemcitabine for 24 or 48 h. The supernatant was then removed and the second compound was added. After an additional exposure period, the second compound was removed and cells were allowed to recover in drug-free medium for 72 h. Growth inhibition was then determined by the MTT assay. For simultaneous exposure, cells were seeded and treated after 24 h with increasing concentrations of sapacitabine alone or with seliciclib, doxorubicin, docetaxel, oxaliplatin or gemcitabine in various concentrations corresponding to the IC₂₀, IC₅₀, IC₇₀ and IC₉₀ values. After approximately four doubling times (120 h), the growth inhibitory effects were measured using the MTT assay.

Statistical analysis and determination of synergistic activity

Drug combination effects were determined using the Chou and Talalay method as described elsewhere (Chou and Talalay, 1984) based on the median effect principle. Combination index (CI) values of < 1 indicate synergy, a value of 1 indicates additive effects and a value of >1 indicates antagonism. Variability between experiments led us to consider that CI values ranging from 0.8 to 1.2 mainly represent additive effects. Thereby calculation of a CI below 0.8 is an indication of synergy, above 1.2 antagonism and between 0.8 and 1.2 an indication of additive effects.

Data were analysed using the concentration effect analysis software (Biosoft, Cambridge, UK). For statistical analysis and graphs, the Instat and Prism software (GraphPad, San Diego, CA, USA) were used. Experiments were performed three times, in duplicate. Means and standard deviations were compared using Student's t-test (two-sided P-value).

Cell cycle analysis and apoptosis

The cell cycle stage and percentage of apoptotic cells were assessed by flow cytometry. In brief, cells were seeded in 25 cm² flasks and were untreated or treated with various concentrations of sapacitabine. At the indicated time points, adherent and non-adherent cells were collected, washed with PBS, fixed in 70% ethanol and stored at 4 °C until use. Cells were re-hydrated in PBS, incubated for 20 min at room temperature (25 °C) with 250 µg ml⁻¹ RNase A with Triton X-100 and 20 min at 40 °C with 50 µg ml⁻¹ propidium iodide in the dark. The cell cycle distribution and percentage of apoptotic cells were determined with FACScan flow cytometer and analysed by FACS Calibur (Becton Dickinson, Le-Ponte-de-Claix, France).

RNA extraction, RT – PCR and quantitative PCR

Expression levels of the various metabolic factors involved in ara-C resistance were assessed by quantitative real-time RT – PCR performed in a LightCycler detection system (Roche, Mannheim, Germany). Briefly, cDNA (5 µl) was mixed with primers (300 nM each), LightCycler-FastStart DNA Master SYBR Green I (hENT1 transporter and POL) or LightCycler-FastStart DNA master hybridisation probes (18S, dCK and cN-II), and probes (130 nM; if necessary) in a total volume of 20 µl. These reactions were prepared in duplicate in three separate experiments. The primer sequences used were:

| Gene   | Forward Primer | Reverse Primer |
|--------|----------------|----------------|
| hENT1  | FOR: gtgggtgtgactgttgat; REV: ctgtagggtggctgtagtg | |
| dCK    | FOR: aacatgagctgtacttccttac; REV: cttgagcttgccattcagaga | |
| cN-II  | FOR: acctgctgtattacccctcgatca; REV: gctccacggttattcagta | |
| POL    | FOR: agctgaccgtgcttgctgct; REV: atagccggagcagacag | |

The relative amount of each target gene and a reference gene (18S) were determined for all samples and the calibrator cell line, HL-60. Ratio results obtained with RelQuant software were considered as final relative PCR arbitrary units. Results were then expressed as PCR arbitrary units in all cell lines related to HL-60 cells PCR arbitrary unit expression.

RESULTS

Single agent studies

The antiproliferative effect of sapacitabine was examined in a panel of 10 cancer cell lines as displayed in Table 1. Time course experiments showed that optimal antiproliferative effects were achieved when cells were exposed to sapacitabine for 48 h. Concentrations of sapacitabine required to achieve an IC₅₀ ranged from 3 ± 0.6 µM for the colon cancer cell line HCT116 to 67 ± 14 µM for the breast cancer cell line MDA-MB-435.

The antiproliferative effects of sapacitabine were compared to those of CNDAC and several commonly used anticancer agents such as gemcitabine, doxorubicin, docetaxel, oxaliplatin and seliciclib. Results are shown in Figure 2. Sapacitabine displayed cytotoxic effects against all cancer cell lines irrespective of the origin of the tumour and usually at lower concentrations than
required for CNDAC. The cytotoxicity profiles of the other nucleoside analogues tested including CNDAC and ara-C appeared to differ from that of sapacitabine suggesting differences in the metabolism, mechanism of action and/or resistance between sapacitabine and other cytidine analogues.

Effect of sapacitabine and CNDAC on cell cycle changes

The HCT116 cell line was selected, as the most sensitive model to both sapacitabine and CNDAC, for further investigation in this study. Cells were incubated with a concentration of 6 μM (twice the IC50) of

Table 1  Antiproliferative effects (IC50 using MTT assay) of sapacitabine, CNDAC, seliciclib and several anticancer compounds in a panel of human and murine cancer cell lines

| Tumour type | Cell line   | Sapacitabine (μM) | CNDAC (μM) | Gemcitabine (μM) | Ara-C (μM) | Seliciclib (μM) | Doxorubicin (μM) | Docetaxel (μM) | Cisplatin (μM) | Oxaliplatin (μM) | 5-FU (μM) |
|-------------|-------------|-------------------|------------|------------------|------------|----------------|------------------|----------------|----------------|----------------|----------|
| Colon       | HT29        | 4.5 ± 0.7         | 160 ± 43   | 0.015 ± 0.003    | 130 ± 40   | 19 ± 3         | 1.07 ± 0.16      | 0.01 ± 0.002   | 25 ± 3         | 60 ± 12        | 24 ± 1   |
|             | HCT116      | 3.0 ± 0.6         | 280 ± 6    | 0.005 ± 0.01     | 1.2 ± 0.4  | 11 ± 2         | 0.10 ± 0.02      | 0.01 ± 0.002   | 9.2 ± 0.9      | 20 ± 4         | 5.3 ± 1.3 |
|             | HCC2998     | 3.5 ± 0.8         | 110 ± 21   | 0.01 ± 0.002     | 3.7 ± 0.5  | 23 ± 5         | 0.70 ± 0.14      | 0.004 ± 0.001  | 25 ± 5         | 4 ± 1.4        | 10 ± 1   |
|             | COLO205     | 5.0 ± 0.8         | 8 ± 2      | 0.2 ± 0.1        | 28 ± 8     | 12 ± 3         | 1.5 ± 0.5        | 0.006 ± 0.001  | 9.5 ± 2.5      | 8 ± 1.6        | 240 ± 20 |
| Breast      | MCF7        | 7.0 ± 1.8         | 850 ± 60   | 0.01 ± 0.002     | > 300      | 16 ± 3         | 0.7 ± 0.1        | 0.01 ± 0.004   | 32 ± 2         | 35 ± 7         | 10 ± 1 |
|             | MDA-MB-435  | 67 ± 14           | 95 ± 13    | 0.04 ± 0.008     | 38 ± 9     | 19 ± 4         | 2.0 ± 0.4        | 0.01 ± 0.002   | 13 ± 2.6       | 37 ± 21        | 10 ± 2 |
| Lung        | HOP62       | 6.0 ± 1.7         | 7.0 ± 1.8  | 0.01 ± 0.002     | 6.3 ± 2.8  | 8 ± 2          | 0.14 ± 0.04      | 0.02 ± 0.04    | 9.3 ± 0.9      | 200 ± 40       | 118 ± 32 |
|             | HOP92       | 3.8 ± 8           | 23 ± 6     | 0.02 ± 0.004     | 1.3 ± 0.3  | 26 ± 4         | 0.10 ± 0.05      | 0.005 ± 0.001  | 4.4 ± 0.4       | 1.4 ± 0.2       | 11 ± 1 |
| Ovarian     | IGROV1      | 6.5 ± 1.6         | 89 ± 12    | 0.02 ± 0.004     | 89 ± 21    | 38 ± 4         | 1.3 ± 0.3        | 0.02 ± 0.01    | 26 ± 6         | 107 ± 21       | 29 ± 1   |
|             | OVCAR3      | 45 ± 7            | > 900      | 1.2 ± 0.2        | > 300      | 20 ± 6         | 1.0 ± 0.2        | 50 ± 0.12      | 1210 ± 3.93    | 50 ± 10         | 24 ± 1 |
| Leukaemia   | L1210       | 20 ± 6            | 1.7 ± 0.3  | ND                | 0.08 ± 0.02 | ND              | ND               | ND             | ND             | ND             | ND     |
|             | L1210dCK-   | 29 ± 4            | 1000 ± 150 | ND                | > 100      | ND             | ND               | ND             | ND             | ND             | ND     |

ND = not determined.

Figure 2  Comparative analysis of cytotoxicity of anticancer agents in a panel of human cancer cell lines. The influence of sapacitabine, CNDAC, seliciclib, doxorubicin, docetaxel, cisplatin, oxaliplatin, ara-C, 5-FU and gemcitabine on the viability of 10 different tumour cell lines was determined using the MTT assay after continuous drug exposure for four doubling times. The indicated values are calculated as follows: log (IC50 individual cell line)–mean (logIC50). Negative values indicate that the cell line is more sensitive than the average, whereas as positive values indicate that the cell line is more resistant than the average. The horizontal axis represents a log scale.
Sapacitabine or CNDAC for 48 h. Cell cycle analysis showed that 35% sapacitabine-treated cells arrested in late-S phase and 41% in G2/M phase. A similar pattern was observed in CNDAC-treated cells with 36 and 36% of cells arrested in late-S and G2/M phases, respectively (Figure 3). Thus, both agents caused the accumulation of HCT116 cells in G2/M phases as reported previously (Azuma et al, 2001).

Analysis of genes involved in nucleoside analogue metabolism by quantitative real-time PCR

The transcription profiles of genes known to be involved in the resistance to nucleoside analogues were analysed in the panel of cancer cell lines. The mRNA levels of hENT1, dCK, CN-II and POL genes were analysed using real-time PCR (Figure 4) and calibrated against HL-60. Colorectal cell lines showed high expression of the transporter hENT1, low-medium expression of the enzyme dCK and a variable expression of the CN-II 5'-nucleotidase and POL enzymes. A similar expression profile was observed in breast cancer cell lines and the HOP92 lung cancer cell line. In contrast, the HOP62 lung cancer cell line and ovarian cancer cell lines (OVCAR3 and IGROV1) showed low expression of the transporter hENT1 and medium expression of CN-II and POL enzymes.

Attempts were made to correlate the antiproliferative effects of sapacitabine, CNDAC, ara-C and gemcitabine with the level of mRNA expression of genes encoding the main drug metabolism proteins. As shown in Figure 5, cells with lower expression of hENT1 and cells with lower expression of dCK appear to be more resistant to both CNDAC and ara-C. In contrast, antiproliferative effects of sapacitabine and gemcitabine were not consistently associated with mRNA levels of hENT1 and dCK. Furthermore, no consistent pattern of expression of CN-II and POL genes correlated with their antiproliferative effects.

Cytotoxicity of sapacitabine in dCK¹/dCK⁻ leukaemia cancer cell lines

The role of dCK in resistance to ara-C has been established with a correlation observed between dCK deficiency and ara-C resistance (Galmarini et al, 2002). As dCK is responsible for intracellular phosphorylation of nucleosides to the active form, we evaluated the cytotoxicity of sapacitabine against the murine leukaemia cell lines L1210 and L1210dCK⁻, the latter was selected on the basis of resistance to continued passage in the presence of ara-C. L1210 cells with dCK activity were sensitive to sapacitabine, CNDAC and ara-C (IC50 20 ± 6, 1.7 ± 0.3 and 0.08 ± 0.02 μM, respectively) (Table 1). We initially confirmed that L1210dCK⁻ cells were indeed resistant to ara-C. L1210dCK⁻ cancer cells were approximately 600- and 2500-fold more resistant to CNDAC and ara-C, respectively, than L1210 cells (IC50 1000 ± 150 and > 100 μM, respectively). However, L1210dCK⁻ cells remained sensitive to the parental compound sapacitabine (IC50 29 ± 4 μM). Together with mRNA profiling, this suggests that sapacitabine alone has antitumour effects based on an intracellular metabolic pathway that may be independent of dCK, which in some cell lines may differ from that of ara-C and its primary metabolite CNDAC. This may be due to the action of the palmitoyl chain of sapacitabine, which is not present in CNDAC.

Combination agent studies

Three schedules were used to study the effect of sequential and simultaneous exposure of combinations of sapacitabine with other anticancer agents. In the first schedule, sapacitabine was added to two cell lines COLO205 and HCT116 for 48 h followed by the addition of oxaliplatin, doxorubicin, docetaxel, gemcitabine or seliciclib for 24 h. The second schedule consisted of inverting the order of exposure of the cells to the different agents, and the third schedule was the simultaneous addition of sapacitabine and a second agent for a period of 48 h. Following incubation, the effect was determined using CIs that represent an affected fraction for the concentration of drugs corresponding to the IC50 as described previously (Chou and Talalay, 1984) (Table 2).

In the docetaxel/sapacitabine combinations, synergistic effects (CI < 1) were observed when docetaxel was given before sapacitabine in both cell lines. Antagonistic effects (CI > 1) were observed for COLO205 when docetaxel was given after and concomitantly to sapacitabine. In the HCT116 cell line, an additive effect was observed for sapacitabine/docetaxel schedule and an additive/synergistic effect in schedule sapacitabine/docetaxel (Table 2). Docetaxel given before sapacitabine appears as more effective in both cell lines than other schedules.

Figure 3  Effects of CNDAC and sapacitabine on cell cycle in HCT116 cells detected by flow cytometry. HCT116 cells were harvested after 48 h of incubation with 6 μM sapacitabine or CNDAC and analysed for DNA content by FACS analysis. The lower panel represents the distribution of cells in different phases of cell cycle.
In the gemcitabine/sapacitabine combinations, synergism was observed in both cell lines with schedule gemcitabine before sapacitabine, in contrast with the antagonistic effect observed when the compounds were administered concomitantly or when sapacitabine was administered before gemcitabine. The synergism observed between sapacitabine and gemcitabine suggests that, although closely related, those two compounds may have distinct mechanisms of action in cancer cells (Table 2). Gemcitabine given before sapacitabine may be more effective than other schedules.

In the doxorubicin/sapacitabine combinations, synergism was observed at high concentrations using sequential exposure, while only additive effects were observed at lower concentrations in both cell lines. Concomitant schedules yielded additive/antagonistic effects (Table 2). Sequential exposure seems to be more effective than combined exposure in both cell lines.

In the oxaliplatin/sapacitabine combinations, various degrees of synergistic activity ($CI < 1$) were observed irrespective of whether sapacitabine was administered before or after oxaliplatin in HCT116. In COLO205 cells, synergy was obtained only when sapacitabine was given concomitantly with oxaliplatin. Therefore, in the colon cancer cell line, COLO205, the effect of the sapacitabine–oxaliplatin combination appeared to be schedule dependent (Table 2).

Combinations of sapacitabine with seliciclib yielded highly synergistic effects for all schedules in the human HCT116 colon cell line, while only additive/antagonistic effects were observed in COLO205 cells. Overall, the optimal schedule appeared to be when seliciclib was given before sapacitabine.

**DISCUSSION**

This study aimed at evaluating the effects of sapacitabine in solid tumour cell lines. The activity of sapacitabine was investigated in a...
demonstrated whether sapacitabine requires to be converted by mechanisms. Although of major interest, it remains to be its bioconversion into CNDAC and may also act by other compounds may suggest that sapacitabine may act both through of the former. However, differences observed between those two may display overlapping cytotoxic effects if the later is a metabolite our study, it is surely not surprising that sapacitabine and CNDAC seliciclib, a novel cell cycle inhibitor currently in clinical trials. In with other anticancer agents in further clinical studies including identifying a pharmacological rational for combining sapacitabine already used anticancer agents. Finally, this study aimed at aiming at comparing cytotoxic effects of sapacitabine with that of ara-C, gemcitabine and CNDAC, as well as based on more pragmatic reasons aiming at comparing cytotoxic effects of sapacitabine with that of already used anticancer agents. Finally, this study aimed at identifying a pharmacological rational for combining sapacitabine with other anticancer agents in further clinical studies including seliciclib, a novel cell cycle inhibitor currently in clinical trials. In our study, it is surely not surprising that sapacitabine and CNDAC may display overlapping cytotoxic effects if the later is a metabolite of the former. However, differences observed between those two compounds may suggest that sapacitabine may act both through its bioconversion into CNDAC and may also act by other mechanisms. Although of major interest, it remains to be demonstrated whether sapacitabine requires to be converted by amidases in vitro or can be directly incorporated into cancer cells, undergoing specific metabolic processes. We observed that sapacitabine displayed antiproliferative activity across a broad range of concentrations in a variety of human cancer cell lines, including cancer cells that were shown to be primarily resistant to several anticancer drugs. Moreover, sapacitabine showed cytotoxic activity against dCK-deficient L1210 cells, indicating a dCK-independent mechanism of action. Finally, sapacitabine showed a synergistic effect when combined with gemcitabine and sequence-specific synergy with doxorubicin and oxaliplatin. Based on these observations, sapacitabine appeared as a good candidate for further evaluation in combination with currently used clinical anticancer agents in tumour types with unmet needs. The antiproliferative effects of sapacitabine in terms of IC\textsubscript{50} values were better than those observed with CNDAC and ara-C. When comparing the cytotoxic effects of these three pyrimidine analogues, sapacitabine appears to retain activity in several cells that were poorly sensitive to CNDAC and ara-C such as colon HT29 and HCC2998, breast MCF7 and ovarian IGROV1. In contrast, gemcitabine achieved cell growth inhibition at lower concentrations. Our study, as well as previously published data, seems to indicate that both CNDAC and sapacitabine are less potent than gemcitabine in cellular and in vivo assays. However this may not turn out to be detrimental in the clinic given that sapacitabine may be given orally and displays additive/synergistic effects with other anticancer agents, allowing maintaining activity in clinical trials. We observed that active concentrations, as assessed by IC\textsubscript{50} values, ranged from 3.0 to 67.0 \textmu M with colon HCT116 and breast MDA-MB-435 being the most sensitive and resistance cancer cells respectively. Based on animal data and preliminary pharmacokinetic information, this range of concentrations appears to be achievable in future clinical trials in humans. Other authors have demonstrated that sapacitabine also had cytotoxic activity against a broad spectrum of human tumour cells, and although the average cytotoxicities were comparable with CNDAC, sapacitabine was more potent (Hanaoka et al, 1999). In gastric, lung, mammary, colon, ovary and epidermoid cell lines, the IC\textsubscript{50} values for sapacitabine ranged from 0.1 to 11 \textmu M, whereas for CNDAC they ranged from 0.1 to > 350 \textmu M (Azuma et al., 1993; Hanaoka et al., 1999; Matsuda and Sasaki, 2004).
Furthermore, sensitivity profiles of dCK-proficient and -deficient cell lines demonstrated resistance to ara-C and CNDAC when dCK is downexpressed. L1210-dCK− cells were approximately 2500- and 600-fold more resistant to ara-C and CNDAC than parental L1210-dCK+ cells, respectively. This resistance was not found when cells were incubated with sapacitabine, suggesting a transport-related mechanism of resistance. This finding showing that sapacitabine retained cytotoxic activity against dCK-deficient L1210 cells strongly suggests that sensitivity to sapacitabine may not only be dependent on dCK expression. The role of dCK in sensitivity to CNDAC and sapacitabine has also been observed in MES-SA-10K, a cell line that derives from MES-SA cell deficient in dCK, selected after repeated passages in the presence of gemcitabine (Galmarini and Green, unpublished observations; Jordeheim et al, 2004). Although dCK-independent mechanisms are not yet finally understood, other enzymes such as CDA have been recently shown to play a role in the mechanism of action of sapacitabine (Fleming et al, 2007). These results support the idea that sapacitabine may emerge as a potent and active agent against dCK-deficient cells.

In our experiments, exposure of HCT116 cancer cells to sapacitabine and CNDAC for 48 h led to the accumulation of cells in G2 phase of cell cycle. The G2 checkpoint is regarded as a resistance mechanism that could permit cells to repair potentially lethal damage such as single-strand breaks. Abrogating this checkpoint reduces the possibility of repair and hence leads to increased cell death. Our data are consistent with the hypothesis that cells treated by sapacitabine or CNDAC may directly progress into G2/M phase of cell cycle. Other authors indicated that after incubation with cytostatic concentrations of CNDAC, cell cycle progression was delayed during S phase, but that cells arrested predominantly in the G2 phase (Azuma et al, 2001; Liu et al, 2005). This unique cell cycle arrest pattern was related to the strand-breaking action of CNDAC caused by the β-elimination-mediated mechanism. The cytotoxic action of CNDAC may be explained by the fact that human tumour cells are able to accumulate and retain CNDACCTP and that the analogue has the ability to terminate further extension.

To further understand the basis of sapacitabine and CNDAC activity in these cell lines of differing origin, we measured mRNA levels of several enzymes commonly implicated in resistance to nucleoside analogues such as dCK, cN-2, POL and hENT1. Colorectal, breast and the HOP92 lung cancer cell lines commonly demonstrated high expression levels of hENT1, low-medium levels of dCK and variable expression of both cN-2 and DNA polymerase, and HOP62 lung and ovarian cancer cell lines showed low expression of the transporter hENT1 and medium cN-2 and DNA polymerase expression; however, no obvious correlation between drug sensitivity and mRNA expression levels of these metabolic factors was identified. Our results are in contrast to those described by Matsuda and Sasaki (2004). These authors demonstrated that levels of expression of metabolic enzymes such as dCK or cytidine deaminase influenced the antiproliferative effects of CNDAC. Moreover, expression and/or function of these factors appear to be influenced by the activation of various oncogenes (Zhang et al, 1999).

To further improve the effects of sapacitabine, circumvent potential resistance to this agent, and find combinations that may be used in clinical trials, we investigated a number of sapacitabine combinations using both simultaneous and sequential exposures to drugs. Combination studies demonstrated synergistic effects when sapacitabine was combined with compounds such as oxaliplatin, gemcitabine, docetaxel and doxorubicin over a broad range of concentrations in human colon cancer cells using several administration schedules. Interestingly, synergy might be sequence-dependent for oxaliplatin and docetaxel. Synergism between sapacitabine and gemcitabine strongly suggests that although closely related these two compounds have distinct mechanisms of action in cancer cells. Phase I clinical data have shown that after oral administration, sapacitabine was not detectable in urine suggesting that most of sapacitabine is converted into CNDAC in human (Delanoit et al, 2006). In our experiments, sapacitabine was consistently more cytotoxic than CNDAC leading to combination studies using sapacitabine instead of CNDAC. Although combination with CNDAC was not

**Table 2** Summary of effects of combination agent study using five anticancer agents in combination with sapacitabine in three treatment schedules

| Combination schedule | COLO205 | HCT116 |
|----------------------|---------|--------|
|                      | ED < 0.25 | ED 0.25–0.50 | ED 0.50–0.75 | ED > 0.75 | ED < 0.25 | ED 0.25–0.50 | ED 0.50–0.75 | ED > 0.75 |
| Docetaxel-based      | 48 h Sapa→24 h Doc | — | — | 1.8 (1.2) | 1.0 (0.6) | 7.4 (5.0) | 0.9 (0.1) | 0.9 (0.4) | 1.3 (0.5) |
| combinations         | 24 h Doc→48 h Sapa | — | 0.5 (0.1) | 1.2 (0.1) | 0.5 (0.3) | 2.1 (1.1) | 0.7 (0.1) | 0.8 (0.1) | 0.1 (0.1) |
|                      | 48 h Sapa+Doc | 1.3 (0.1) | 1.5 (0.7) | 0.9 (0.2) | 0.5 (0.1) | — | 1.5 (0.1) | 0.7 (0.3) | 0.7 (0.4) |
| Gemcitabine-based    | 48 h Sapa→24 h Gem | 1.4 (0.5) | 1.0 (0.1) | 1.0 (0.4) | 1.2 (0.8) | 2.6 (0.9) | — | 0.7 (0.1) | 1.3 (0.8) |
| combinations         | 24 h Gem→48 h Sapa | 1.5 (0.3) | 1.0 (0.3) | 0.5 (0.1) | 0.4 (0.2) | — | 0.4 (0.1) | 0.5 (0.3) | 0.4 (0.3) |
|                      | 48 h Sapa+Gem | 6.0 (0.20) | 1.7 (0.8) | 1.0 (0.3) | 0.6 (0.5) | — | — | 1.3 (0.9) | 0.4 (0.2) |
| Oxaliplatin-based    | 48 h Sapa→24 h Oxa | — | — | — | 0.6 (0.5) | — | 1.1 (0.2) | 0.8 (0.4) | 0.7 (0.5) |
| combinations         | 24 h Oxa→48 h Sapa | — | — | — | 0.7 (1.1) | 0.8 (0.1) | 0.8 (0.3) | 1.1 (0.5) | 0.6 (0.6) |
|                      | 48 h Sapa+Oxa | 2.0 (1.9) | 1.2 (0.9) | 0.7 (0.7) | 1.9 (0.9) | 1.9 (0.1) | 1.0 (0.3) | 1.2 (0.2) | 1.0 (0.3) |
| Seliciclib-based     | 48 h Sapa→24 h Sel | — | 2.2 (1.1) | 1.8 (0.5) | 1.3 (0.5) | 8.0 (1.2) | 0.4 (0.1) | 0.3 (0.1) | 0.6 (0.4) |
| combinations         | 24 h Sel→48 h Sapa | 1.2 (0.3) | 1.4 (0.1) | 0.9 (0.2) | 0.6 (0.1) | 1.8 (0.6) | 0.5 (0.4) | 0.5 (0.4) | 0.4 (0.4) |
|                      | 48 h Sapa+Sel | 0.7 (0.6) | 0.8 (0.4) | — | 2.1 (0.9) | 1.1 (0.2) | 0.7 (0.2) | 0.7 (0.7) | 0.7 (0.7) |

CI = combination indices; Doc = docetaxel; Oxa = oxaliplatin; Sel = seliciclib.
performed, it is unlikely that results may have been superior with CNDAC than that of sapacitabine in our models.

In summary, our study provides additional data supporting further preclinical and clinical pharmacological investigations of sapacitabine in human cancers. Our results suggest that sapacitabine possesses an interesting toxicity profile compared to several other anticancer agents including other pyrimidine analogues. Moreover, sapacitabine showed cytotoxic activity against dCK-deficient cell lines indicating that this compound can be active in cells resistant to CNDAC, ara-C and other nucleoside analogues. Our preclinical data showed that sapacitabine combinations yield synergistic activity with other anticancer agent (including gemcitabine) supporting further evaluation of those combinations both simultaneously and sequentially in clinical trials.

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REFERENCES
Azuma A, Huang P, Matsuda A, Plunkett W (2001) 2'-C-cyano-2'-deoxy-1-beta-D-arabino-pentofuranosylcytosine: a novel anticancer nucleoside analog that causes both DNA strand breaks and G(2) arrest. Mol Pharmacol 59: 725–731
Azuma A, Nakajima Y, Nishizono N, Minakawa N, Suzuki M, Hanaoka K, Kobayashi T, Tanaka M, Sasaki T, Matsuda A (1993) Nucleosides and nucleotides. 122.2'-C-cyano-2'-deoxy-1-beta-D-arabino-pentofuranosylcytosine and its derivatives. A new class of nucleoside with a broad antitumor spectrum. J Med Chem 36: 4183–4189
Chou TC, Talalay P (1984) Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enz Regul 22: 27–55
Delaunoit T, Burch PA, Reid JM, Camoriano JK, Kobayash T, Braich TA, Kaur JS, Rubin J, Erlichman C (2006) A phase I clinical and pharmacokinetic study of CS-682 administered orally in advanced malignant solid tumors. Invest New Drugs 24: 327–333
Fairev S, Raymond E, Woynarowski J, Cvtikovic E (1999) Supradditive effect of 2,2'-difluorodeoxycytidine (gemcitabine) in combination with oxaliplatin in human cancer cell lines. Cancer Chemother Pharmacol 44: 117–123
Fleming IN, Gudmundsdottir E, Sleigh R, Green SR (2007) Identification of biomarkers and drug combinations to aid clinical development of sapacitabine (CYC682), a novel nucleoside analogue. 2007 AACR Annual Meeting. Proc Am Assoc Cancer Res; abstract 3138, p. 756
Galmarini CM (2006) Drug evaluation: sapacitabine – an orally available antimetabolite in the treatment of cancer. Curr Opin Investig Drugs 7: 565–573
Galmarini CM, Mackey J, Dumontet C (2002) Nucleoside analogues and nucleobases in cancer treatment. Lancet Oncol 3: 415–424
Hanaoka K, Suzuki M, Kobayashi T, Tanazawa F, Tanaka K, Shibayama T, Hanaoka K, Tanazawa F, Kurakata S, Kobayashi T, Sasaki T, Matsuda A, Tsudo T, Kurakata S (1999) Antitumor activity and novel DNA-self-strand-breaking mechanism of CNDAC (1-(2-C-cyano-2'-deoxy-beta-D-arabino-pentofuranosyl) cytosine) and its N4-palmitoyl derivative (CS-682). Int J Cancer 82: 226–236
Hansen M, Nielsen S, Berg K (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 119: 203–210
Jordhein L, Gros E, Gouy MH, Galmarini CM, Peyrottes S, Mackey J, Perigaud C, Dumontet C (2004) Characterization of a gemcitabine-resistant murine leukemia cell line: reversion of in vitro resistance by a mononucleotide prodrug. Clin Cancer Res 10: 5614–5621
Kaneko M, Koga R, Murayama K, Shibata T, Hotoda H, Suzuki M, Hanaoka K, Tanazawa F, Kurakata S, Kobayashi T, Sasaki T, Matsuda A (1997) Synthesis and antitumor activity of a novel antitumor nucleoside 1-(2-C-cyano-2'-deoxy-B-D-arabino-pentofuranosyl)-N4-palmitoylcytosine (CS-682) - Eighty-Eighth Annual Meeting of the American Association for Cancer research. Proc Am Assoc Cancer Res 38: 101 abstract
Liu X, Guo Y, Li Y, Jiang Y, Chubb S, Azuma A, Huang P, Matsuda A, Hittelman W, Plunkett W (2005) Molecular basis for G2 arrest induced by 2'-C-cyano-2'-deoxy-1-beta-D-arabino-pentofuranosylcytosine and consequences of checkpoint abrogation. Cancer Res 65: 6874–6881
Matsuda A, Sasaki T (2004) Antitumor activity of sugar-modified cytosine nucleosides. Cancer Sci 95: 105–111
McClue SJ, Blake D, Clarke R, Cowan A, Cummings L, Fischer PM, MacKenzie M, Melville J, Stewart K, Wang S, Zhelev N, Zheleva D, Lane DP (2002) In vitro and in vivo antitumor properties of the cyclin dependent kinase inhibitor CYC202 (R-roscovitine). Int J Cancer 102: 463–468
Meijer L, Raymond E (2003) Roscovitine and other purines as kinase inhibitors. From starfish oocytes to clinical trials. Acc Chem Res 36: 417–425
Mu W, Mazurchuk R, Chaudhary ND, Sperryak J, Veith J, Pera P, Greco W, Hoffman RM, Kobayashi T, Bernacki R (2003) High-resolution magnetic resonance imaging of the efficacy of the cytosine analogue 1-(2-C-cyano-2'-deoxy-beta-D-arabino-pentofuranosyl)-(N4)-palmitoyl cytosine (CS-682) in a liver-metastasis athymic nude mouse model. Cancer Res 63: 2477–2482
Zhang M, Endo Y, Sasaki T (1999) Determinants in chemosensitivity of oncogene-transformed NIH3T3 cells to 2'-C-cyano-2'-deoxy-1-beta-D-arabino-pentofuranosylcytosine. Int J Onco 14: 543–549