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Research and identification of polymorphic variants of the gene of human carboxylesterase 2, an enzyme involved in the activation of irinotecan into SN-38

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Irinotecan is widely used in the treatment of metastatic colorectal carcinoma. It is a prodrug requiring hepatic activation to the topoisomerase I - interactive drug, SN-38. We had shown earlier a wide individual variation of irinotecan activation by human liver microsomes. Plasma levels of SN-38 also vary in large proportions in patients treated with irinotecan. Carboxylesterase 2 (hCE2) is the enzyme which is mostly responsible for irinotecan activation. There is a possibility that a genetic polymorphism of this enzyme explains the individual variability of SN-38 production in humans and, therefore, some of the variability of the therapeutic and toxic effects of irinotecan in patients. The carboxylesterase 2 gene (16q22.1) contains 12 exons. Human genome sequence in public databases has listed 6 single nucleotide polymorphisms (SNP) in the gene, but no data is yet available about their frequency in the population and their possible role on the expression and activity of the enzyme. None of these variations is able to alter protein sequence, but one of them is located in the promoter of the gene, at position -332 before the transcription initiation codon. In a first attempt, we have explored in a population of 11 individual human DNA samples the existence of allelic variants (i) at the level of each exon and at the intron-exon junctions; (ii) at the level of the 6 SNPs already mentioned in the databases (promoter, intron 2, intron 10, intron 12). This has been done using denaturing high performance liquid chromatography (dHPLC) of 14 different fragments amplified by PCR and sequencing of the amplified fragments which had a modified profile. We have identified a total of 6 allelic variations which constitute three different haplotypes in addition to the wild-type sequence. These variations occurred at the level of the promoter, intron 10 and intron 12 (12a and 12b). We have then extended this study to 53 human DNA samples using dHPLC of the 4 fragments where an alteration had been detected. We have quantified the frequency of the 3 haplotypes in this population: haplotype *1 (promoter, intron 10, intron 12b): 9.4%; haplotype *2 (intron 10): 5.7%; haplotype *3 (intron 10, intron 12a, intron 12b): 11.3%. Another SNP was identified in exon 12 at position 1703, associated with the SNP in intron 10, constituting a 4th haplotype with a frequency of 1.9% and no alteration of protein sequence. A study of the whole gene is ongoing on the 53 DNA samples.

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New structural analogues of lycobetaine with high anticancer activity *in vitro*

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The phenanthridine alkaloid lycobetaine, a minor constituent of Amaryllidaceae, showed very good anti-tumor activity *in vitro* and was found to act as a specific topoisomerase IIβ poison (BJC, 2001, 85(10):1585-91). Here we report on the antitumor activity of 25 lycobetaine analogues, as well as 11 synthesis intermediates and closely related compounds. In a first step, inhibition of tumor growth was studied in a cellular proliferation assay in a panel of 12 human tumor cell lines comprising colon, central nervous system, gastric, non-small cell lung, mammary, melanoma, renal and uterus cancer cell lines. Three compounds (E758, E759, and E761) showed a better antitumor activity than the lead compound lycobetaine (IC_{50} = 6.9 μM). The mean IC_{50} values of the three lycobetaine derivatives were 0.46 μM (E761), 1.2 μM (E759), and 1.4 μM (E758). Compared to the activity of established anticancer agents acting via topoisomerase inhibition, the novel derivatives were similarly potent to etoposide (IC_{50} = 1.0 μM). Lycobetaine, E758, E759 and E761, as well as etoposide were further studied in the clonogenic assay, using 28 human tumor xenografts growing subcutaneously in nude mice. Again, the three derivatives of lycobetaine were more active than the lead compound lycobetaine. The mean IC_{50} values of the three derivatives were 2.0 μM (E761), 3.3 μM (E758), and 4.4 μM (E759), compared to a mean IC_{50} of 11.3 μM of lycobetaine. Regarding the fingerprint profiles of the compounds over 28 human tumor xenografts in the clonogenic assay, the most sensitive tumors were the melanoma MEXF 989, the uterus carcinoma UXF 1138, and the renal cancers RXF 944 and RXF 631 followed by the lung model LXFA 526 and the mammary model MAXF 401. In conclusion, 3/36 analogues of lycobetaine showed a higher antitumor activity than the lead compound lycobetaine. Further *in vivo* studies in nude mice in human tumor xenografts found as sensitive in the clonogenic assay are warranted.

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Pharmacokinetic and Pharmacogenetic Analysis of Irinotecan (CPT-11) Given in Combination with R115777

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The topoisomerase I inhibitor CPT-11 is a prodrug of the pharmacologically active metabolite SN-38, and is approved for the first-line treatment of advanced colorectal cancer. Recently, we conducted a phase I study in which we combined this drug with the farnesyltransferase inhibitor R115777 (Zarnestra'). The maximum tolerated dose was established at 300 mg BID R115777 administered orally for 14 consecutive days q3weeks in combination with 350 mg/m^2 CPT-11 i.v. (90 minutes) q3weeks. The pharmacokinetics (PK) of CPT-11 and SN-38 were determined during 2 courses in a total of 27 patients. In the first course, R115777 was started 2 days after the infusion of CPT-11. In the second course, it was given together. Blood samples were analyzed by HPLC. Next, a recently developed population-PK (NONMEM) model for CPT-11 (Xie et al, J Clin Oncol, 2002) was used to calculate the kinetics of the lactone, carboxylate and total forms of CPT-11 and its metabolite. As patients were treated on different dose-levels of both CPT-11 (200-350 mg/m^2) and R115777 (200-400 mg BID), a dose-normalization was performed. As both anti-cancer agents are partly metabolized by the detoxifying enzyme UGT1A1, a pharmacogenetic analysis for polymorphic variants in UGT1A1*28 was done, using pyrosequencing. In the first course, the mean AUC for the total forms of CPT-11 and SN-38 were 17,763 $\pm$ 4,234 ng.h/ml and 476 $\pm$ 181 ng.h/ml, respectively. In the second course, the CPT-11 AUC was 12.5% higher (paired, 2-sided Student t-test: P = 0.004) with a value of 19,980 $\pm$ 5,309 ng.h/ml), whereas the SN-38 AUC increased by 18%, with a mean value of 562 $\pm$ 300 ng.h/ml (P = 0.036). Also, most other parameters (AUCs and clearances for the lactone and carboxylate forms) of both compounds differed significantly between both courses. The genetic code for UGT1A1 could be determined in 22/27 patients (12 wild-type patients, 9 heterozygous for an extra TA repeat and 1 homozygous for the same variant). No apparent relation between this polymorphism and the PK of CPT-11 could be discovered. In conclusion, a substantial increase in the systemic exposure to CPT-11 and the active metabolite SN-38 was observed following continuous administration of R115777. This may be due to inhibition of UGT1A1, which could lead to less glucuronidation of SN-38. However, the clinical relevance of these findings remains to be established, since both drugs could be safely given in combination at their full-recommended single agent dose.

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Pilot studies of antimetabolites preceeded by irinotecan in advanced solid tumors

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In a Ward rat colon color carcinoma (ca) model, 5-fluorouracil (FUra) showed 95% complete tumor regression (CTR) when irinotecan (IRN) was given 24 h before, but only 38% when it was given 24 h after FUra (50% of MTD of each agent). At these doses, FUra alone gave 25% CTR and IRN gave zero (Cao and Rustum, Cancer Res 60:3717, 2000). A fourfold reduction in the dose of IRN led to only a minor decrease in activity, indicating that IRN is acting as a modulator; preliminary data indicated that IRN caused cells to accumulate in S-phase leading to potentiation of FUra activity. This effect is being explored in two pilot studies in patients (pts) with advanced solid tumors A: IRN/FUra and B: IRN/Gemcitabine (GEM). All drugs are given intravenously. IRN is given over 1.5 h, followed 24 h later by FUra 400 mg/m^2 over 0.08 h, weekly $\times$ 4 q 6 weeks (A) or by GEM 1000

mg/m^2 over 0.5 h, weekly × 2 q 3 weeks (B). In A, downward dose modification in successive cohorts based on the results of recruitment of cells into S-phase in tumor biopsies (measured by immunohistochemistry for Cyclin A and flow cytometry (FC)) before and after IRN, is used to determine the minimal modulatory dose of IRN. Starting dose (SD) was 80mg/m^2, with subsequent exploration of 40 and 60 mg/m^2 (present dose). In B, doses are escalated on the basis of toxicity in standard Phase I fashion with 3 pts at each dose level not showing dose limiting toxicity (DLT). Three patients have been entered at each of the doses 20, 40 and 60 mg/m^2 (present dose). Pre and post IRN biopsies are performed in some pts. Toxicities > grade 3, with cycle 1 include neutropenia grade 3-4 (6 pts) and hyponatremia (1 pt). Cyclin A index (S+G2) increased by 95-200% (mean 134%) in 4 evaluable pts by IHC and S phase by 265% and 128% in 2 evaluable pts by FC, at 80 mg/m^2 of IRN. At 40 mg/m^2, increases in Cyclin A index were seen in 2/4 evaluable pts but these increases were <50%. Data indicate marked modulation of cell cycle at a dose of 80 mg/m^2 but not at 40 mg/m^2 of IRN. Correlation of cell cycle modulation with pharmacokinetics of IRN and SN-38 are in progress.

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Histone deacetylase inhibitors potentiate breast cancer cell lines to anthracycline-induced apoptosis in a schedule and dose-dependent manner

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Anthracyclines are thought to act by binding to topoisomerase IIá (topo IIá). Covalent binding will stabilize the topo IIá:DNA complex and there by inhibit synthesis of nucleic acids and proteins, resulting in subsequent cell death. Sensitivity to topo IIá inhibitors is dependent on theexpression level of topo IIá in cancer cells. Increased expression of topoIIá has been associated with sensitivity to topo IIá inhibitors. Histone deacetylase inhibitors (HDAC-I) such as sodium butyrate have been shown toincrease topo IIá. The HDAC-I, suberoylanilide hydroxamic acid (SAHA), hasbeen shown to inhibit growth and promote differentiation and apoptosis inseveral transformed cell lines including breast cancer. SAHA is now in earlyclinical trials and appears well tolerated. While HDAC-I may have anti-tumor activity as single agents, synergistic and antagonistic activity has been reported when combined with cytotoxic agents according to preclinical studies. In this study, we examined the effects of SAHA on apoptosis induced by topo IIá inhibitors. We found that cultured breast cancer cells with high levels of topo IIá (e.g.SKBr-3) were sensitive to anthracyclines resulting in growth arrest and apoptosis, whereas epirubicin caused minimal apoptosis in cells with low topo IIá levels (e.g.MCF-7). In SKBr-3 cells, SAHA enhanced epirubicin-induced apoptosis. However, synergistic activity was only observed when cells were preexposed to SAHA for at least 48 hours. Synergistic and additive effects were abrogated when SAHA was administered simultaneously or after exposure to epirubicin. Sensitization correlated with alteration of topo IIá. Furthermore, in cells with low topo IIá, SAHA-pretreatment resulted in sensitization even at concentrations that showed no significant apoptosis by the anthracycline alone. The topo IIá inhibitors, doxorubicin and epirubicin are currently amongst the most active agents for the treatment of breast cancer, but many tumors are resistant to this therapy. Our *in vitro* studies show that the combination of HDAC-I and epirubicin significantly enhances the activity of these agents and/or overcomes resistance in cells with low levels of topo IIá.

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Specific inhibition of the growth-associated alpha isoform of topoisomerase II by the novel anticancer triazoloacridone C-1305

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Triazoloacridones are new antitumor compounds with potent activity against human leukemias as well as toward solid tumors in animal models. The most active triazoloacridone derivative C1305 is able to stimulate the formation of covalent DNA-topoisomerase II complexes *in vitro*. Topoisomerase II exists as two different isoforms: the alpha isoform which is restricted to proliferating tissues and which frequently is upregulated in human tumors and the beta isoform which is constitutively expressed in all tissues. Unexpectedly, only the alpha isoform of topoisomerase II was covalently associated with DNA following exposure of living cells to C-1305 in marked contrast to amsacrine, a classical topoisomerase II inhibitor which stimulated cleavable complexes with both isoforms. Pulse-field electrophoresis revealed, that the exposure of tumor cells to C-1305 resulted in the formation of 50-200 kbp fragments which correspond to the size of DNA loops. In contrast, amsacrine induced the formation of fragments of about 1,000 kbp. To further clarify the relative importance of the two topoisomerase isoforms in the cytotoxic action of C-1305, different sublines of the DC-3F Chinese hamster cells were studied. Prolonged ellipticine exposure of DC-3F parental cells led to selection of resistant DC-3F/9-OHE cells which have no expression of the beta form and 5-fold decreased expression of the alpha isoform. In addition, two transfectants were used where the expression of one of the two isoforms had been restored by transfection of the resistant cell line with either the alpha or the beta form of topoisomerase II. In agreement with the results presented above, the cytotoxicity of amsacrine was increased in cells transfected with either isoform, while the sensitivity to C-1305 was only altered in cells transfected with the alpha isoform. These results identify C-1305 as the first topoisomerase II inhibitor which selectively targets the growth- and tumor-associated alpha form of topoisomerase II.

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Phase I and pharmacologic study of the macromolecular topoisomerase-I-inhibitor DE-310 given once every 2 or 6 weeks in patients with solid tumors

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DE-310 is a novel macromolecular drug-delivery system, which comprises the topoisomerase-I inhibitor DX-8951 linked to a biodegrable carrier, carboxymethyldextran polyalcohol, via a peptide spacer. In pre-clinical studies, it has been demonstrated that DE-310 accumulates and is retained preferentially in tumor tissue by an enhanced permeability and retention effect. In the current Phase I study, DE-310 was initially administered as an IV infusion over 3 hours once every 2 weeks (q2w) in a 4-week cycle. Resulting pharmacokinetic (PK) data indicated that the apparent half-life of DE-310 approximated the 2 weeks administration interval, and so the protocol was amended to a 6 weekly schedule (q6w). Dose levels tested to date include: q2w; 1 mg/m^2 (dose level I, n=6), 2 mg/m^2 (dose level II, n=4) and q6w; 6 mg/m^2 (dose level III, n=3), 9 mg/m^2 (dose level IV, n=3). Currently, 16 patients (9 male, 7 female), median age 59 years (range, 31-78), median PS 1 (range, 0-2), with a variety of refractory solid tumors are included. The worst hematologic toxicities are: grade 4 neutropenia (n=1), grade 4 leucopenia (n=1), grade 3 thrombocytopenia (n=2), and grade 3 anemia (n=1), all at dose level IV. Non-hematological toxicities are mild to moderate, including: nausea, vomiting, diarrhea, fatigue, anorexia, alopecia, skin reaction, and infusion reaction. At dose level IV, 1 patient experienced DLT due to febrile neutropenia and grade 3 thrombocytopenia. Out of 15 patients assessable for response, 9 achieved stable disease and 2 have major objective tumor regression, which formally qualify as partial responses. To evaluate the PK of DE-310, conjugated DX-8951, DX-8951 and G-DX-8951 were measured in samples taken up to 35 days post first dose. At dose level III, the apparent half-life of conjugated DX-8951, DX-8951 and G-DX-8951 were 13, 10 and 11 days respectively. The AUC0-t ratio (conjugated DX-8951/DX-8951) was approximately 600 and the mean Tmax of DX-8951 was achieved at 75 hours post dose. The active moiety DX-8951 declined in parallel with the carrier-linked molecule, conjugated DX-8951, suggesting that DX-8951 elimination is formation rate-limited. Overall, these data indicate that DX-8951 is released slowly and over an extended period, achieving the desired prolonged exposure. The observed tumor regressions are of interest.