44

Cytochrome P450 3A4 mediates transformation of methoxymorpholinyl doxorubicin (Nemorubicin; MMDX) to its highly potent metabolite PNU-159682 in human liver microsomes

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MMDX is a promising doxorubicin (DX) derivative currently undergoing clinical investigation for treatment of hepatocellular carcinoma. Despite an *in vitro* cytotoxicity comparable or slightly (2-10-fold) higher than that of DX, MMDX is 50-100-fold more potent than DX *in vivo* both in experimental animals and in humans. Furthermore, our previous *in vivo* studies carried out in the mouse model suggest that an MMDX metabolite(s) possessing a higher potency than the parent compound and synthesized via cytochrome P450 3A (CYP3A) mediates MMDX activity *in vivo* (Quintieri et al., Cancer Res.; 60: 3232, 2000). Among the known metabolites of MMDX, only PNU-159682, a compound we previously isolated from an incubation mixture of the drug with NADPH-fortified rat liver microsomes, shows significantly higher *in vitro* cytotoxicity compared with MMDX; its biosynthesis may therefore explain the high potency of MMDX *in vivo*. The aims of this study were to evaluate the ability of human liver microsomes (HLM) to catalyze conversion of MMDX to PNU-159682 and to identify the enzyme(s) involved. Preliminary studies using 14C-MMDX and radio-HPLC analysis indicated that biotransformation of MMDX by HLM is an NADPH-dependent process and that PNU-159682 represents the major liver microsomal metabolite of the drug. Further experiments were conducted using HPLC with fluorescence detection for quantitative evaluation of metabolite formation. Kinetic analyses indicated that a single enzyme supports conversion of MMDX to PNU-159682. CYP3A4 appears to be responsible for PNU-159682 synthesis based on the following key results: 1) among the CYP inhibitors tested, only TAO (100 μM) and ketoconazole (1 μM), both of which are selective inhibitors of CYP3A enzymes, strongly inhibit PNU-159682 formation (85 and 95% inhibition, respectively); 2) metabolite formation is dose-dependently inhibited by a monoclonal anti-CYP3A4/5 antibody; 3) using 10 individual preparations of human liver microsomes, we observed a highly significant correlation (r2 = 0.97) between the rate of PNU-159682 formation and testosterone 6 β-hydroxylase activity, a marker of CYP3A; 4) only microsomes from CYP3A4 cDNA-transfected lymphoblastoid cells but not those from cells engineered to express other human liver CYP proteins are able to catalyze the formation of detectable amounts of the metabolite. In conclusion, PNU-159682 represents a CYP3A4-generated hepatic metabolite of MMDX in humans.

45

Preclinical activity against liver metastases of Nemorubicin, a DNA-intercalating cytotoxic agent for the treatment of hepatocellular carcinoma

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Nemorubicin (3'desamino-3'[2(S)methoxy-4-morpholinyl]doxorubicin-hydrochloride, PNU-152243) is a highly lipophilic anthracycline currently under clinical development for the intra-hepatic artery treatment of hepatocellular carcinoma. This anticancer drug is biotransformed in the liver into a more cytotoxic metabolite(s) by means of a cytocrome P4503A (CYP3A)-dependent process, as shown *in vitro* by incubating the drug with NADPH-fortified liver microsomes (Quintieri et al, 2000). The metabolism of Nemorubicin is not fully elucidated; at present one metabolite exhibiting higher potency compared to the parent compound has been identified (Geroni et al, 1997). The metabolic potentiation of Nemorubicin by hepatic microsomal enzyme(s) may contribute to rendering this drug highly active against primary liver tumors or liver metastases. A phase I study reported regressions in patients with liver metastases from colorectal cancer (Vasey et al, 1995). We studied the activity of Nemorubicin in comparison to doxorubicin (DX) in C57BL/6 mice bearing tumors derived from the cell line M5076, which preferentially metastasize to the liver. Unlike DX, Nemorubicin is more effective against liver metastases than against the primary solid tumor following i.v. administration. Results showed an increase in lifespan (ILS) of 59% and 67% for Nemorubicin and 63% and 39% for DX on primary tumor and metastases, respectively. Moreover, Nemorubicin shows higher antimetastatic activity following oral administration (ILS, 107%); this finding supports a role of an active metabolite(s) synthesized via intestinal and/or hepatic enzyme(s) in its activity toward tumor cells growing in the liver. The role of CYP3A-mediated Nemorubicin metabolism on its *in vivo* antitumor activity and toxicity has been tested. Pretreatment of mice with the prototypical inducers of CYP3A, pregnenolone-16β-carbonitrile (PCN) and dexamethasone (DEX), increases liver microsomal potentiation of the *in vitro* cytotoxicity of Nemorubicin. Furthermore, pretreatment of animals with DEX reduces Nemorubicin toxicity, while not interfering with its antitumor activity. Conversely, administration of troleandomycin, a selective inhibitor of CYP3A activity, markedly inhibits the antitumor activity of Nemorubicin and increases drug tolerability. Collectively, these findings suggest that a Nemorubicin active metabolite(s) synthesized by CYP3A contributes significantly to its antitumor activity, mainly at the hepatic level.

46

Cellular pharmacology of PNU-159682, a liver microsomal metabolite of methoxymorpholinyl doxorubicin (Nemorubicin; MMDX)

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PNU-159682 is a major liver microsomal metabolite of MMDX, a doxorubicin (DX) derivative currently undergoing a phase II/III clinical trial. The purposes of this work were to obtain preliminary information about the cytotoxicity, effects on cell cycle progression, and DNA-binding properties of PNU-159682. In a first set of experiments we compared the *in vitro* cytotoxicity of PNU-159682 with that of MMDX and DX toward a panel of eight tumor cell lines including three human leukemias (EM-2, Jurkat and CEM), a human colon adenocarcinoma (HT-29), a human ovarian carcinoma (A2780), a human prostatic carcinoma (DU145), a murine melanoma (B16F10) and a murine leukemia (L1210). Each tumor cell line was exposed to the drugs for 1 h and then cultured in drug-free medium for 72 h. Drug concentrations that decreased cell growth by 70% (IC70) were calculated from dose-response curves by linear interpolation. The IC70 values of PNU-159682 ranged between 0.07 and 0.58 nM; these values were considerably lower than that recorded for both MMDX (67.6-577.9 nM) and DX (181.0-1,717.3 nM). Further experiments analyzed the cell cycle perturbations induced in LoVo colon adenocarcinoma cells by exposure to equitoxic concentrations of each drug. Cells were continuously exposed to the IC70 value of each compound recorded after 72 h of drug treatment, and the cell cycle distribution profiles analyzed using flow cytometry. Exposure to PNU-159682 determined an accumulation of the cells in the S or G2/M phase at 24 h; in contrast, MMDX and DX induced a G1 and a G2/M arrest, respectively. Between 24 and 48 h of treatment a small number of cells started to undergo apoptosis as revealed by the presence of a sub-G1 peak at 48 h. This phenomenon became more evident at 72 h of drug exposure. Preliminary DNA unwinding studies showed that PNU-159682 is able to intercalate into DNA with a higher affinity than MMDX and DX. In conclusion, the results described indicate that PNU-159682 is a compound endowed with a much higher cytotoxicity than MMDX and DX. Furthermore, the different effect of PNU-159682 on cell cycle progression and its higher DNA binding affinity, compared with that of MMDX and DX, suggest that it might possess a different molecular mechanism of action.

47

Properties of the new anthracycline derivative containing modified daunosamine moiety

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The anthracycline antibiotics such as daunorubicin, doxorubicin, epidoxorubicin are widely used drugs in the treatment of variety of human neoplastic diseases. However, their clinical effectiveness is limited by several factors, including dose-dependent cardiotoxicity. We have found that during synthesis of new derivatives of daunorubicin with amidine group in 3' position, a new compound (AOX) containing oxazoline ring in daunosamine

moiety was formed. The structure of that one was confirmed by 1H and 13C NMR and IR spectra. Advantageous influence of such modification in daunosamine moiety on the biological properties of AOX, as compared to parental daunorubicin, was observed. Lethal toxicity of AOX is considerably lower (about 20 times). The LD_{50} (lethal dose 50%) of AOX in mice approaches 64.0 mg/kg, whereas the LD_{50} of daunorubicin -3,1 mg/kg. Preliminary histological studies on the cardiotoxicity have shown that cardiotoxicity of AOX is significantly lower than that of parental daunorubicin. It should be stressed that AOX, in contrary to referential daunorubicin, is able to overcome the barrier of drug resistance in *in vitro* conditions. The resistance index (RI) for AOX (ID_{50} value against the cells of anthracycline resistant cell subline divided by respective value against the cells of drug sensitive parental line) is about 1. These results show that antiproliferative activity of AOX against drug sensitive and drug resistant cells is very similar. Moreover, compound AOX revealed the antiproliferative activity *in vitro* against the cells of various mouse and human cancer cell lines and antitumor activity *in vivo* in P 388 mouse leukemia model similar or even higher than referential daunorubicin. Modification in daunosamine moiety (AOX) appeared to cause also important changes in the physicochemical properties. We have observed that stability of AOX during storage in different temperatures as well as in organic solutions is higher than those of parental daunorubicin. It has to be pointed out that the observed results evidently show that such significant modification of the structure of daunosamine moiety in daunorubicin molecule (AOX) does not decrease its antiproliferative activity but induces the advantageous changes of its biological and physicochemical properties such as decreased lethal toxicity and cardiotoxicity, increased stability as well as the ability to overcome the barrier of drug resistance.

48

The combination of nemorubicin with cisplatin and mitomycin C is synergistic in experimental tumor models

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Nemorubicin (3'desamino-3' [2(S)methoxy-4-morpholinyl] doxorubicin-hydrochloride, PNU-152243) is a doxorubicin derivative currently undergoing clinical investigation for the treatment of hepatocellular carcinoma. The compound is characterized by high potency and broad spectrum of antitumor activity, including activity on multidrug resistant (MDR) models, with respect to other anthracyclines. Combination studies have been performed considering both the mode of action of nemorubicin and its ability to overcome MDR (both p-glycoprotein and topoisomerase II related MDR mechanisms) and alkylating agent resistance. Moreover, tumor cells selected for resistance to nemorubicin are specifically resistant to morpholinylanthracyclines and sensitive to all other antitumor drugs. Interestingly, nemorubicin-resistant tumors show a remarkable collateral sensitivity to cisplatin and mitomycin C, with no increase in general toxicity. The model chosen for combination studies is the disseminated L1210 murine leukemia. Mice are treated with nemorubicin (i.v. on day 1 and 2 post tumor implant), or cisplatin or mitomycin C (i.v. on day 3), or their combination. Results: At the highest not-toxic dose (HNTD, 0.06 mg/kg/dose, total dose of 0.12 mg/kg), nemorubicin administered as a single agent shows a 67% increase in life span (ILS). The HNTD of cisplatin alone (10 mg/kg) or mitomycin C alone (6 mg/kg) presents 50% or 0% ILS, respectively Clear synergy is obtained at the highest non toxic combination of nemorubicin (0.06 mg/kg/dose) and cisplatin (10 mg/kg) with a 133% ILS. Synergy is retained on 2 additional lower dose levels. Synergistic antitumor effect is also observed when nemorubicin is combined with mitomycin C. The highest non-toxic combination doses of nemorubicin (0.04 mg/kg/dose) and mitomycin C (4 mg/kg) show a 93% ILS. In conclusion, these data provide a rationale for further clinical trial using the administration of nemorubicin in combination with cisplatin and mitomycin C.

Bioreductive agents

49

A role for over-expressed human cytosolic NOS-II in the bioactivation and toxicity of tirapazamine *in vitro*

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Tirapazamine (TPZ) is a lead member of a class of bioreductive drugs currently in Phase II and III clinical trials. Recent studies have suggested that TPZ radicals that are formed outside the nucleus do not contribute to intranuclear DNA damage and that metabolism that occurs in the cytoplasm is probably irrelevant for activity of this drug in producing DNA-damaging radical. In this study, we have investigated the role of over-expressed cytosolic inducible nitric oxide synthase (NOS II) in the bioactivation of this DNA-damaging antitumour agent. NOS is a dimeric enzyme providing two catalytic domains: an oxygenase and a reductase domain. The reductase domain shares a high degree of sequence homology with cytochrome P450 reductase, known to bioactivate TPZ. To achieve this, we have constitutively over-expressed human NOS-II activity in the breast cancer cell line MDA 231 by employing an optimised expression vector in which the strong human polypeptide chain elongation factor 1 alpha promoter drives a bicistronic message containing the genes for human NOS II and the puromycin resistant gene (pac). In transfected cells, subcellular localization following differential centrifugation and nuclei isolation, showed NOS-II catalytic activity was elevated within the cytoplasm and was exclusively soluble, as determined by conventional activity assay. Both confocal microscopy and Western Blotting studies were carried out to confirm our findings. NOS reductase activity as measured by the NADPH-dependent reduction of cytochrome c ranged from 7.5 to 22.0 nmol cyt. c reduced/min/mg. The highest activity represented about 8-fold increase over parental activity. Similarly, the catalytic activity of the oxygenase domain, measured by the conversion of 14C-labelled L-arginine to L-citrulline, ranged from 20 to 66 pmol citrulline / min/mg and the highest activity was about 110-fold over parental cell activity. NADPH-dependent metabolism of TPZ to the stable two-electron metabolite, SR4317, was determined by HPLC analysis. Increase in rate of metabolism mirrored the elevation in NOS II reductase expression. More significantly, sensitivity to TPZ following 3 hr hypoxic exposure also increased with elevated NOS II reductase levels. Taken collectively, our findings are of significant importance since they indicate that cytosolic NOS II-derived tirapazamine radicals may be important in enhancing its cellular toxicity through DNA damage.

50

A phase I study of fenretinide combined with paclitaxel and cisplatin for the treatment of refractory solid tumors

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Fenretinide (F) is a semi-synthetic retinoid with antiproliferative activity in preclinical studies. F is able to induce apoptosis in a variety of cell types, independent of the expression of retinoid receptors, and appears to function in receptor mediated and non-receptor mediated pathways. Cisplatin (C) and paclitaxel (P) have broad activity alone and in combination in a variety of solid tumors. F has a distinct and interesting mechanism of action and demonstrates more than additive activity when combined *in vitro* with a variety of agents including C and P. The objectives of this study were to determine the maximum tolerated doses and clinical toxicity of C/P/F when used in combination in patients with advanced cancer, and to determine a recommended phase II dose of these agents together. In addition, we characterized the pharmacokinetics (PK) of these agents when administered together and documented responses in patients. All patients provided informed consent for this NCI and IRB approved protocol. C/P were given in standard fashion on day 2, and F was given orally in 2 divided daily doses for 8 days, starting 24 hours prior to C/P. Cycles were repeated every 3 weeks for a maximum of 6 cycles. PK was obtained on all 3 drugs on day 2 (following C/P) and day 8 (following the last dose of F) during cycle one. 15 patients (mean age 57.3, range 26-77) were enrolled on the study, 14 were assessable for PK, toxicity and response. One patient was removed prior to completing the 1st cycle of treatment as he developed acute cholecystitis. The remaining 14 patients received between 1 and 6 cycles of treatment (with 2 patients currently receiving treatment). Dose limiting toxicity (Gr 3 diarrhea and Gr 4 neutropenia) was encountered in 2/4 patients at 80/175/1800 mg/m^2 C/P/F. Seven patients received 2-6 cycles at the next (recommended) dose level of 60/135/1800. NCI CTC 2.0 grade 3 and 4 toxicities included fatigue, nausea/vomiting, neuropathy, and dehydration. 2 patients had a major response (NSCLC and squamous esophageal) and 4 patients had stable disease for up to 6 cycles. A major issue of tolerability was the number of 100 mg pills required to achieve the recommended dose of F. Many patients were unable to swallow the prescribed 26+ daily pills due to nausea or other factors. Compliance with F dosing ranged from 53-100%. PK analysis is underway at the time of submission. (Supported by U01-CA76576-02 and P30-CA16058).