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Abstracts

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ANTIPROLIFERATIVE EFFECTS OF FERROCENIUM ORGANOMETALLIC COMPLEXES

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Different authors have been proposed the ferrocene derivate (Fe^{II}-$\eta^5[C_5H_5]_2$) and its corresponding ferrocenium salts (Fe^{III}-η^5 $[C_5H_5]_2^+)X^-$ as antiproliferative agents. These molecules showed a direct correlation between antitumor activity and the effects produced on DNA. In this study we have evaluated the effects and mechanism of action of the original ferrocenium salt decamethyl-ferrocenium tetrafluoroborate ($DEMFc^+$) in comparison with four other ferrocenium tetrafluoroborate salts (ferrocenium; 1,1'-dimetylferrocenium; ferrocenium-boronic acid and ferrocenium-carboxylic acid) and with ferrocene. The chemical stability in aqueous buffered solutions was measured by UV-vis spectroscopy. The cellular effects of the compounds were determined in MCF-7 tumor cell line. We measured the antiproliferative effects, assayed by dye exclusion and MTT tests, and the amount of oxidative damages on DNA, determined with flow cytometry analysis (FACS) for the specific fluorescent probe 8-oxo-guanine. We have confirmed with electronic spin resonance (ESR) measurements that these cationic compounds react in buffer saline producing oxygen radical species.
$DEMFc^+$ showed the highest cytotoxic effect (i.e., IC_{50} 37±4 microM) and dose- and time-dependent antiproliferative effects, in a 5 – 100 microM range. This complex showed a dose-dependent ability to produce oxidative DNA damages in a concentration range of 10^{-5}-10^{-6} M. Our data demonstrate that ferrocenium derivate molecules posses antiproliferative effects based on their capacity to generate oxygen active species. This radical mechanism of action of these complexes is likely related to the initial oxidation state (III) of their central iron atom and exerts its effects by producing DNA damages.

DEVELOPMENT AND VALIDATION OF A NEW HPLC-FLUORIMETRIC METHOD TO DETERMINE EPIRUBICIN AND ITS METABOLITE EPIRUBICINOL IN DOG PLASMA

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Epirubicin (EPI) is a semisynthetic derivative of doxorubicin, an anthracycline widely used in human medicine as anticancer drug. Cats and dogs are frequently affected by mammary cancers. In this disease, surgical treatment has an elective role but, in the few past years, chemotherapy has progressively substituted the old invasive method. Since some drugs are highly toxic, monitoring drug concentrations during therapy is widely recommended to avoid severe adverse effects. The present study describes a rapid and simple HPLC method, validated according to the EMEA guidelines on validation of analytical procedures, to detect EPI and epirubicinol (EPIol) in dog plasma. Four mongrel female dogs, 3 - 4 years hold, 20-25 kg, were administered 2 mg/kg IV EPI. Blood was collected at different times and plasma was extracted. The mobile phase consisted in NaH_2PO_4 50mM / CH_3CN 65:35 v/v pH 4.00, the detector was set at 480 nm and 560 nm for excitation and emission, respectively. The flow was 1 ml/min, attenuation 0.004 AUFS, column was Spherisorb ODS2 C18 150 mm length. 500 µl of dog plasma were mixed with internal standard (daunorubicin 10 µg/ml) added with 500 µl Na_2HPO_4 0.2 M and shacked with 3 ml of $CHCl_4$-CH_3OH 90:10 v/v for 20 minutes. After centrifugation, the organic phase was picked up and extraction repeated twice. The organic layers were collected and dryed. 250 µl of CH_3OH were added and 20 µl were injected onto HPLC. The LOQ and LOD for EPI and EPIol were 10 and 5, 1 and 0.5 ng/ml, respectively. Recovery was 85.5±2.5 % (SF) for both substances. Precision and accuracy were within 10 %. Reproducibility was assessed by means of inter-laboratory trials. EPI concentration reached a plateau after 30 min, while EPIol concentration was under LOQ for the first 30 min and rose to Cmax (47 ng/ml) after 4 hours. 8 Hours after the administration, EPI was under LOQ, while EPIol was detected also after 56 hours. As EPIol seems to be the main responsible of cardiotoxicity, this method could be used to asses the EPI and EPIol plasma concentrations in dog plasma to avoid the severe adverse effects due to this anticancer-drug.

EFFECTS OF COMBRETASTATIN ANALOGUES ON LEUKEMIC CELL LINES AND NORMAL HEMOPOIETIC CELLS

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Combretastatins are natural antimitotic agents isolated from the bark of the South African tree Combretum caffrum. Among these compounds, combretastatin A-4 possesses an interesting anticancer activity. With the aim to find new combretastatine derivatives endowed with potent cytotoxic activity on cancer cells we synthesized two new series of combretastatin (CA-4) analogues. The alkenyl motif of CA-4 was replaced either by five-membered heterocyclic (isoxazoline or isoxazole) or by six-membered ring (pyridin or benzene). The new compounds have been evaluated for their effects on tubulin assemblay and for cytotoxic and apoptotic activities on different normal and leukemic cell lines. Five compounds demonstrated an attractive profile of cytotoxicity ($IC50<1\mu M$) and apoptosis-inducing activity but poor antitubulin activity. In contrast to CA-4 that caused a block of cells in M phase of cell cycle, the isoxazoline derivatives caused a block in G2, suggesting that these compounds could act on targets different from the mitotic spindle. Interestingly, isoxazoline derivatives were active in apoptosis resistant and BCR-ABL expressing K562 cells (blastic crisis of chronic myelogenous leukemia) and in multidrug resistant (MDR) HL60-R and K562-ADR cells. These compounds showed a low cytotoxicity on normal hemopoietic stem cells. Isoxazoline derivatives caused a marked disruption of the mitochondrial potential $\Delta\Psi$ and apoptosis induced by these derivatives was, in part, inhibited by the caspase-9 inhibitor Z-LEHD. Moreover, the apoptotic activity of isoxazoline derivatives was decreased in presence of the Fas-blocking antibody ZB4. This indicates that these derivatives trigger apoptosis activating both the mitochondrial pathway and the Fas/Fas-L pathway. In conclusion, our data suggest that structural alteration of the stilbene motif of CA-4 can be

ANTITUMOR EFFECTS OF CURCUMIN, ALONE OR IN COMBINATION WITH CISPLATIN, ON HEPATIC CANCER CELLS. ANALYSIS OF THEIR RELATIONSHIP TO IL-6 PRODUCTION

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Curcumin (CUR), a polyphenolic compound extracted from rhizomes of *Curcuma* species, is endowed with potentially interesting anti-inflammatory and anti-tumor properties. We have observed that CUR can exert significant growth inhibitory and apoptotic effects in different human hepatocellular cancer (HCC) cell lines, like the HepG2, Huh-6 and HA22T/VGH ones. Further, CUR sensitized HA22T/VGH cells to the anti-tumor and apoptotic effects of cisplatin. Since interference of CUR with autologous IL-6 production by tumor cells may contribute to explain these effects[1], we investigated in detail this aspect. At difference of HepG2 or Huh-6, by ELISA determinations, HA22T/VGH showed to secrete IL-6 in huge amounts (16.8 ng /10^6 cells/24 h). Curcumin actually decreased this production, by down-regulating, likely through inhibition of constitutive NF-kB expression and in a caspase-independent way, IL-6 mRNA. Flow cytometry analyses showed that HA22T/VGH cells express the gp130 subunit of the IL-6 receptor at their surface. For the IL-6 receptor α subunit, the cells showed to produce the mRNAs of both its membrane and soluble form. However, the corresponding proteins were neither expressed at the cell surface nor secreted, as shown by flow cytometry and ELISA assays. In MTT assays, as expected, incubation with a neutralizing anti-IL-6 antibody for up to 7 days did not significantly affect the growth of HA22T/VGH cells or sensitize them to cisplatin. Also treatment with an effective anti-IL-6 antisense oligodeoxynucleotide failed to produce this result. Overall, inhibition of an autocrine IL-6 loop does not appear to explain the direct antitumor effects of CUR in the HA22T/VGH model of HCC. However, since release of IL-6 by tumor cells appears to be frequent in HCC, especially in its more advanced stages[2], CUR administration in this tumor might beneficial also to contrast the adverse systemic effects (e.g. cachexia) of the cytokine.

[1] Chan M.M., Fong D., Soprano K.J., Holmes W.F. and Heverling H. (2003) J. Cell Physiol.

BIOLOGICAL EVALUATION OF COMBRETASTATIN ANALOGUES

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Combretastatin A4 is an inhibitor of tubulin polymerization that has recently entered clinical trials as an antitumoral agent. Indeed, this agent has been shown to display a selectivity for neovascular formations, thereby causing ischemia in the neoplastic structure. Combretastin A4 is poorly soluble in water and has therefore entered clinical trials as a phosphate salt.
Chemically, combretastatin is a relatively simple molecule, with two substituted phenyls linked by an olefin bridge. The olefin bridge can exist in both cis and trans conformations, and only the former is biologically active. In light of this, we have synthesised and tested a series of compounds that present a furazan ring bridging the two phenyl groups. In principle, this modification should lock the molecule in the cis conformation. Furthermore, we have also attempted to generate hybrid molecules that contain both combretastatin and nitrogen mustards.
To test these compounds, we used SH-SY5Y neuroblastoma cells as a model, and analysed cytotoxicity, tubulin polymerization, caspase-3 activation, and cell cycle modifications. In this model combretastatin was a potent cytotoxic agent, with an IC_{50} of 1.5±0.28 nM. Although most of the compounds synthesised lost significant potency compared to combretastatin, we found that cis-locked compounds presenting a *meta* amino or hydroxyl on ring B displayed a 3-fold increase in potency compared to combretastatin. Similarly, we found that bridging combretastatin and chlorambucil with an ester chain yielded a compound displaying increased potency compared to combretastatin. All these compounds acted by inducing apoptotic cell death, as shown by caspase-3 activation , by producing a selective G2/M block and inhibiting microtubule polymerization.

ANTITUMOR EFFECTS OF THE GRAPE STILBENES RESVERATROL AND PICEATANNOL ON MULTIDRUG AND APOPTOSIS RESISTANT HL60 LEUKEMIA CELLS

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The grape stilbenes trans-resveratrol (RES) and trans-piceatannol (PIC) have promising anticancer and other therapeutic properties. PIC differs from RES for an additional hydroxyl group and probably represents an active metabolite of RES in humans. Since few studies have directly compared the antitumor activities of RES and PIC, we examined their effects in the HL60 leukemia and, as an especially new issue, in its multidrug resistant variant HL60R. HL60R overexpresses P-glycoprotein (P-gp) and also the principal Inhibitory of Apoptosis Proteins [1]. RES and PIC exhibited dose-dependent growth inhibitory effects (as shown by MTS or cell viability assays), associated with induction of cell death (examined by flow cytometry analyses of DNA fragmentation), in both cell lines; interestingly, HL60 was someway more sensitive to RES (in MTS assays, the IC_{50} of RES after 48 h was 35.5 µM and that of PIC 61 µM) and HL60R to PIC (RES and PIC IC_{50} were of 86 and 47 µM, respectively, in this cell line). P-gp did not appear to influence these activities of the compounds, since co-incubation with the P-gp inhibitor verapamil did not significantly change their antitumor effects in either cell line. Further, RES or PIC exerted additive or sub-additive antitumor effects when combined with the P-gp substrate doxorubicin in HL60R or HL60. Finally, treatments with the thiol donor N-acetyl cysteine or with the inhibitor of glutathione synthesis buthionine sulfoximine reduced or increased, respectively, mainly the antitumor effects of PIC, rather than of RES, in the cell lines. Thus, a pro-oxidant mechanism may be relevant in PIC antitumor activity in HL60 and HL60R cells. In conclusion, these results underline the antileukemic activity of the grape stilbenes, also suggesting that their use, in particular that of PIC, might be of benefit also in conditions of multidrug and apoptosis resistance. Further experiments are needed to see whether the present results may have a wider applicability and to better explain their mechanistic aspects.

[1] Notarbartolo M., Cervello M., Dusonchet L., Cusimano A. and D'Alessandro N. (2002) Cancer Lett. 180: 91-101

ROLE OF STAT3 IN THE RESPONSE OF HUMAN BREAST CARCINOMA CELLS TO DOXORUBICIN

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Signal transducer and activator of transcription (STAT)-3 is reported to play a role in oncogenic transformation and to be constitutively activated in a number of malignancies. Interestingly, some of the genes modulated by STAT3 encode antiapoptotic factors (Bcl-x_L, Bcl-2), suggesting an involvement of STAT3 in drug resistance. In the present study, we evaluated (a) whether the response of human breast cancer cells (HBCCs) to the cytotoxic agent doxorubicin (DOX) is affected by STAT3 activation; and (b) whether STAT3 downregulation can sensitize HBCCs to the action of this drug. STAT3 protein levels (both in baseline conditions and following DOX treatment) were determined by immunoblotting in four HBCC lines (MCF-7, SK-BR1, MDA-MB-231 and MDA-MB-468). STAT3 activation was determined by measuring the phosphorylation state of the protein and its nucleocytoplasmic ratio. The antiproliferative and proapoptic effects of DOX were evaluated by the MTT assay and by the TUNEL reaction followed by flow cytometric analysis, respectively. The following IC_{50} values were obtained upon 72h exposure to DOX: MCF7 359.29±17.61 nM; MDA-MB-231 503.56±51.52 nM; MDA-MB-468 163±19.29 nM; SKBR-3 77.14±5.09 nM (Mean±SD of 6-9 independent experiments). No correlation was found between IC_{50} values and baseline expression/activity of STAT3; rather, DOX cytotoxicity seemed to be inversely related to the ability of the different HBCCs to upregulate STAT3 following treatment, indicating that STAT3 activation could be an early response to drug treatment, aimed at promoting cell survival and resistance. When such activation was prevented by co-incubating the cells with the tyrphostin AG490, inhibiting the catalytic activity of JAK kinases that act directly upstream of STAT3, the response to DOX was potentiated in some HBCCs. We conclude that downregulation of STAT3 could represent a novel approach to HBCC chemosensitization to DOX.

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ON THE REDUCED CARDIOTOXICITY OF EPIRUBICIN: FROM GLUCURONIDATION TO IMPAIRED CONVERSION TO TOXIC SPECIES IN HUMAN HEART

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Clinical use of the anticancer anthracycline doxorubicin (DOX) is limited by cardiomyopathy upon chronic administration. 4'-Epidoxorubicin (epirubicin, EPI) is credited with reduced cardiotoxicity, reportedly because glucuronidation by hepatic UDP-glucuronosyltransferase 2B7 facilitates EPI elimination in bile and urine. We considered that EPI cardiotoxicity could be limited also by its inherent resistance to reductive bioactivation to toxic species like secondary alcohol metabolites and reactive oxygen species (ROS). Because animal models of anthracycline metabolism are limited by potential pitfalls we assessed DOX and EPI metabolism in human myocardium. Small myocardial biopsies were obtained from patients undergoing aorto-coronary bypass grafting and exposed to 1-10 µM EPI or DOX, concentrations similar to those found in patients' plasma shortly after boluses of either drug. Anthracycline metabolites were measured by HPLC, and ROS were measured by adapting the dichlorofluorescein assay to studies in whole tissues. DOX and EPI exhibited similar uptake and accumulation in human myocardium (nmol/g wet wt/4h : 1.7±0.2 *vs* 2.1±0.2 and 13.9±0.4 *vs* 15.8±1 for DOX *vs* EPI at 1 µM or 10 µM, respectively). Secondary alcohol metabolites were detected only at 10 µM anthracycline; however, EPI formed less alcohol metabolite than DOX (nmol/g wet wt/4 h : 0.03±0.002 *vs* 0.06±0.002; n=6, P< 0.001). Kinetic studies showed that this was due to an impaired affinity of EPI for cytoplasmic reductases [V_{max}/k_m (mL/mg prot^{-1} min^{-1}): 1.1×10^{-5} *vs* 1.8×10^{-4} for EPI *vs* DOX). DOX increased the myocardial levels of ROS by 93±43% and 179±62% at 1 and 10 µM, respectively; in contrast, EPI had no activity at either concentration. Subcellular fractionation showed that this was due to a limited access of EPI to mitochondrial reductases. These results show that EPI exhibits altered cellular trafficking and reduced conversion to toxic species in human heart, and serves guidelines for improving clinical use of EPI beyond "the more glucuronidation-the less cardiotoxicity" concept. Moreover, the model adopted in this study is suitable for screening the metabolic determinants