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**Molecular alterations of KIT and PDGFR-alpha; in GISTs – A Portuguese experience**

A.L. Gomes[1], A. Gouveia[2,3,5], A.F. Capelinha[2,4,5], D.D.L. Cruz[4], P. Silva[2,4,5], R. Reis[1], A. Pimenta[3,4,5], J.M. Lopes[2,4,5]. *[1]ICVS-University of Minho, School of Heatl Sciences, Braga, Portugal; [2]IPATIMUP, Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal; [3]H. S. João, Department of Surgery, Porto, Portugal; [4]H. S. João, Department of Pathology, Porto, Portugal; [5]University of Porto, Medical Faculty of Porto, Porto, Portugal*

**Background:** GISTs are rare primary mesenchymal tumours of the gastrointestinal system. Detection of Kit overexpression by immunohistochemistry is a hallmark of GIST diagnosis. Kit is a type III receptor tyrosine kinase, belonging to the PDGFR family. Kit overexpression usually results from activating mutations in the extracellular domain (exon 9), juxtamembrane domain (exon 11), and intracellular kinase domains (exons 13 and 17) of *KIT* oncogene. Approximately 80% of *KIT* mutations occur in exon 11, and are predictive of high sensitivity response to tyrosine kinase receptor inhibitor, Imatinib®. *KIT* wild-type GIST cases may harbour alternative activating mutations of *PDGFR-α,* hotspot region (exons 12, 14 and 18), in a mutually exclusive way. The frequency of *KIT* mutations ranges from 20 to 80% of GIST reported cases. In Portugal, the incidence of *KIT* and *PDGFR-α* mutations in GISTs is virtually unknown. The aim of this work was to assess the frequency of *KIT* and *PDGFR-α* oncogenes mutations in a Portuguese series of GISTs.
**Material and Methods:** Eighty-one formalin-fixed and paraffin-embedded *bonafide* primary previously untreated GISTs were selected after clinical-pathological review. Mutation analysis of *KIT* exon 11, as well as of *PDGFR-α* (exon 12, 14 and 18) was done by direct sequencing. Analysis of *KIT*, exons 9, 13 and 17 was done by PCR-SSCP, followed by direct sequencing.
**Results:** Of the 81 GISTs, 38 (47.0%) contained mutations in exon 11, 4 (4.9%) in exon 9, and 1 (1.3%) in each exon 13 and 17 of *KIT*. Mutations in exon 11 included deletions (44.7%), duplications (2.6%), base substitutions (36.8%), and mixed mutations (15.8%), i.e. deletions together with either insertions or base substitutions. All but one mutation in exon 9 were the same duplication (502dup503). All deletions and duplications were in-frame. Alterations in exon 13 and 17 were silent mutations. Of *KIT* mutation negative cases, *PDGFR-α* mutations were present in 3/18 (16.7%) in exon 12, 5/29 (17.2%) in exon 18, and none in exon 14 [0/15 (0%)]. Mutations in exon 12 and 18 included deletions (25%) and base substitutions (75%). Twenty (24.7%) GISTs were "wild-type" for both oncogenes. In eight *KIT* wild-type bearing GISTs, *PDGFR-α* was not possible due to available material. No correlation was found between mutations of both *KIT* and *PDGFR-α* oncogenes and clinical-pathological features (e.g. risk group, tumour size, and location); lack of *KIT* mutation was statistically associated ($p < 0.0197$) with epithelioid GISTs.
**Conclusions:** The incidence of mutually exclusive *KIT* (54.3%) and *PDGFR-α* (10.9%) mutations in this Portuguese GISTs series fits with published series, namely of South European Countries. Epithelioid GISTs usually do not harbour *KIT* mutations. As reported, the great majority of *KIT* mutations 38/44 (86.4%) are located in exon 11, indicating a favourable response to Imatinib-based therapy.

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**Time dependent effects of Gefitinib on ABCG2: modulation of CPT-11 efficacy in colon cancer cell lines**

A. Azzariti, L. Porcelli, G. Simone, S. Gagliardi, A. Paradiso. *National Cancer Institute, Clinical Experimental Oncology Lab, Bari, Italy*

Recent data indicate that ABCG2 displays a high-affinity interaction with several tyrosine kinase receptor inhibitors, including Gefitinib, which seem to be responsive of an increase sensitivity to camptothecins. These data suggest a crucial role of this pump in multidrug therapy, with the combination of EGFR inhibitors with camptothecins. Our previous study concerning the possibility to combine gefitinib and SN-38 (the active metabolite of CPT-11) put in evidence that the two drugs were synergic or antagonist in function of the schedule utilised; in fact, when SN-38 was given before gefitinib, they sinergically increased cell growth inhibition conversely, in the opposite schedule they were antagonist.
In order to explain the opposite effects of the two sequential schedule of gefitinib plus SN-38 and to investigate the involvement of ABCG2 in these phenomena, we analysed the capability of the TK inhibitor to modulate ABCG2 expression in function of time, by western blot analysis, and as a consequence, the modulation of SN-38 intra-cell accumulation after the cell pre-exposure to gefitinib, by HPLC analysis. Moreover, we analysed the capability of gefitinib to modulate SN-38 activity, as cell cycle perturbation, after short gefitinib exposure (1hour) and in function of drug concentration, by flow cytometry. In this study, we utilised gefitinib at a sub-active concentration (IC30) and SN-38 at the IC50 concentration in two colon cancer cell lines, LoVo and HT-29.
Our results showed that ABCG2 expression was modulate by gefitinib only after prolonged exposure, with a stimulation from 5 to 14 days conversely, at short time (from 15 min to 3 days) it was not affected. These evidences suggest to determine SN-38 accumulation after 5 days gefitinib exposure and it was strongly reduced, justifying the antagonism between the two drugs on cell growth. Moreover, the analysis of cell cycle modulation by short time gefitinib exposure (1day) showed that the cell accumulation in S-phase, when the two drugs were given together or gefitinib before SN-38, was reduced as respect the SN-38 alone and with a gefitinib concentration-dependency conversely, the synergic schedule, SN-38 followed by gefitinib showed an increased S-phase accumulation. In this report and for the first time, we provide evidences that gefitinb could act as an ABCG2 inhibitor, when given for short time, as already reported by other authors, and as a stimulator of this pump after prolonged exposure These preliminary results confirmed that the interaction between gefitinib and ABCG2, in function of time exposure, could be a very relevant factor necessary to consider when a multidrugs therapy is planned.

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**Sphingolipids enhance expression of the multidrug resistance phenotype in human breast cancer cells**

V. Gouaze-Andersson, J. Yu, A. Kreitenberg, A. Giuliano, M. Cabot. *John Wayne Cancer Institute, Santa Monica, California, USA*

**Background:** Although overexpression of P-glycoprotein (P-gp) by the multidrug resistance gene (MDR1) is one of the most consistent alterations associated with cancer cell resistance to chemotherapy, lipids such as ceramide also affect response to chemotherapy. For example, many natural-product anticancer agents enhance cellular levels of ceramide, a proapoptotic sphingolipid. Because high levels of the ceramide metabolite glucosylceramide (GC) often coincide with elevated P-gp, we hypothesized that ceramide and GC might influence expression of the multidrug resistance phenotype.
**Materials and Methods:** Three wild-type human breast cancer cell lines (MDA-MB-435, MDA-MB-231, T47D) were exposed for several days or several passages to short-chain, cell-permeable ceramide (C8-cer) or glucosylceramide (C8-GC). MDR1 expression (mRNA) was determined by real-time RT-PCR (beta-actin internal control); expression of P-gp was determined by Western blot. Cell sensitivity to chemotherapy was evaluated by viability assays in 96-well plates, and cell efflux capacity was assessed by using rhodamine-123.
**Results:** Short-term (3-day) exposure to C8-cer (5.0 ug/ml media, 11.7 uM) enhanced MDR1 mRNA levels by 3-fold and 5-fold in T47D and MDA-MB-435 cells, respectively. Growth of MDA-MB-231 cells with C8-cer for extended periods enhanced MDR1 expression by 45-fold and 370-fold at passages 12 and 22, respectively. This was accompanied by an increase in P-gp levels, enhanced rhodamine efflux, and a decrease in cellular sensitivity to chemotherapy (doxorubicin, paclitaxel). Short-term (3-day) treatment with C8-GC (10 ug/ml media) increased MDR1 expression by 4-fold in MDA-MB-231 cells as compared with glycolipid-naïve controls. Exposure of cells to octanoic acid, a C8-cer hydrolysis product, or to oleic acid (18:1) did not alter MDR1 expression.
**Conclusions:** Here we show for the first time that ceramide and glycolipids upregulate MDR1 expression in breast cancer cells. This suggests that ceramide's role as a messenger of cytotoxic response to chemotherapy is linked to the multidrug resistance pathway. Targeting GC synthesis might circumvent resistance to natural product chemotherapy.

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**THPC11PtCl2: a novel platinum compound that overcomes cisplatin resistance by inducing a different mechanism of apoptosis**

A. Dietrich[1], T. Mueller[1], W. Voigt[1], B. Kalinowski[2], R. Paschke[2], H.-J. Schmoll[1]. *[1]Martin-Luther-University of Halle, Hematology/Oncology, Halle, Germany; [2]Martin-Luther-University, Medical-Pharmaceutical Chemistry, Halle, Germany*

*Background:* Despite cisplatin (CDDP)-based chemotherapy are curative in testicular germ cell tumors (TGCT), the development of platinum drugs with improved antitumoral activity continues to be a productive field of research. Particularly with regard to circumvent CDDP resistance we designed a new platinum derivative THPC11PtCl2, consisting of cisplatin linked to a tetrahydropyrane via an aliphatic C11-spacer. The purpose of the present work was to compare the cytotoxic potential of this drug with CDDP using the CDDP-sensitive TGCT-cell line H12.1 and the CDDP-resistant TGCT-cell line 1411HP.

*Material and methods:* Cytotoxic activity (IC50 and IC90) was tested by colorimetric SRB assay. In subsequent assays cell lines were treated with equitoxic IC90 doses of THPC11PtCl2 and CDDP. Drug uptake studies were performed using atomic absorption spectroscopy. Determination of apoptotic cell death was proven by DNA fragmentation and cleavage of PARP. Regarding the regulation of apoptosis induction, activation of caspase-3 was examined by western blotting and substrate cleavage kinetics. Furthermore treated and untreated cells were investigated for cytochrome c release, p53 expression and cell cycle analysis. Representing the induction of structural alterations of DNA cells were subjected to DNA-gelelectrophoresis.
*Results:* The cell line 1411HP showed a 3.3-fold CDDP-resistance as compared to H12.1 by respective IC90 values, which could completely overcome by treatment of cells with THPC11PtCl2. Measurements of platinum uptake revealed a higher accumulation of THPC11PtCl2 as compared to CDDP in both cell lines. Moreover THPC11PtCl2 was 2-fold more enriched in resistant 1411HP than in H12.1. Treatment with both agents resulted in a similar release of cytochrom c and cleavage of caspase-3 and PARP. However, after exposure to THPC11PtCl2 activation of caspase-3 was accelerated and no upregulation of p53 was observed. In addition, pre-treatment with the caspase inhibitor Z-VAD-Fmk did not inhibit apoptosis induction. THPC11PtCl2 treatment led to a DNA-mobility different from CDDP and induced no cell cycle arrest.
*Conclusions:* Our results revealed a selective higher activity and an increased drug accumulation of THPC11PtCl2 in the CDDP-resistant TGCT-cell line 1411HP. Considering the different efficacy and mechanism of apoptosis induction bypassing the caspase- and cell cycle-dependent apoptotic pathway, THPC11PtCl2 could serve as a new promising anti-cancer agent.

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**A phase II study of daily gefitinib plus weekly paclitaxel (GP) in Taiwanese non-small cell lung cancer (NSCLC) patients who failed prior gefitinib or both gefitinib and taxane treatment**

C. Tsai, C. Chiu, C. Lai, J. Liu, R. Perng. *Taipei Veterans General Hospital, Chest Department, Taipei, Taiwan*

**Background:** Gefitinib (G) is an active agent in 27% of Taiwanese NSCLC patients. GP has demonstrated anticancer activity in patients who failed G.
**Purpose:** To evaluate the activity and toxicity of GP in NSCLC patients who had previously failed treatment with G or with both G and taxane (G/T).
**Methods:** Eligibilities were histologically and/or cytologically proven NSCLC failed G (refractory or resistant), measurable lesion, ECOG PS0–3, adequate organ function, life expectancy longer than 6 weeks and written informed consent. They were chemonaive or had failed prior chemotherapeutic regimen(s). Oral G (250 mg) daily and intravenous P (60 mg/m$^2$) d1, 8, 15 were administered and repeated every four weeks. Primary endpoint was response rate (RR) and secondary endpoints were disease control rate (DCR), time to progression (TTP), overall survival (OS) and toxicity.
**Results:** From Sep 2004 to Sep 2005, 33 pts were enrolled and deemed eligible: M/F 16/17; median age 64; PS 1/2/3 13/17/3; stage IIIB/IV 3/30; adeno/squamous/NS 25/3/5. All 33 patients failed G, of them 4 were chemonaive, 8 had prior G and non T agents, 21 had prior G/T (docetaxel 13, paclitaxel 11), and 24 had prior platinum. Median prior chemotherapy regimen was 2 (0–7). A total of 281 cycles (median 4, range 1–18) were given. RRs were 24% and 29%, DCRs 48% and 43%, TTP 96 days and 45 days, median OS 264 days and 182 days for the overall and G/T groups, respectively. Grade 3/4 toxicities were leukopenia 6% and 5%, anemia 3% and 5%, Grade 3 flu-like symptoms 30% and 30% in overall and G/T groups, respectively. There was one treatment-related death.
**Conclusion:** This GT regimen showed a moderate activity and low toxicity as salvage treatment in patients who previously failed G or G/T treatment.

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**Combined modalities of resistance in an oxaliplaitin-resistant human gastric cancer cell line with enhanced sensitivity**

L.-T. Chen[1], C.-C. Chen[1], T.-C. Tsou[2], W.-Y. Pan[1], C.-C. Kuo[1], J.-F. Liu[1], S.-C. Yeh[2], F.-Y. Tsai[2], H.-P. Hsieh[3], J.-Y. Chang[1]. *[1]National Health Research Institutes, Institute of Cancer Research, Taipei, Taiwan; [2]National Health Research Institutes, Division of Environmental Health and Occupational Medicine, Taipei, Taiwan; [3]National Health Research Institutes, Division of Biotechnology and Pharmaceutical Research, Taipei, Taiwan*

**Background:** In order to identify mechanisms underlying oxaliplatin resistance, a subline of the human gastric adenocarcinoma TSGH cell line was made resistant to oxaliplatin by continuous selection against increasing drug concentrations. In present study, we would like to investigate the biochemical and molecular mechanisms through which cells acquire oxaliplatin resistance.
**Materials and Methods:** The *in vitro* $IC_{50}$ and $LC_{50}$ values were examined by the methylene blue dye assay and clonogenic survival assay, respectively. GSH/GSSG assay, platinum accumulation assay, platinum-DNA adduct assay, host cell reactivation assay, RNA interference technique, RT-PCR, and Western blotting were used to reveal molecular events in this study.
**Results:** Compared with the parental TSGH cells, the S3 subline showed 58-fold resistance to oxaliplatin; it also displayed 11-fold and 2-fold resistance to cis-diammine-dichloroplatinum (II) (cisplatin, CDDP) and copper sulfate, respectively. Interestingly, S3 cells were 4-fold more susceptible to 5-fluorouracil-induced cytotoxicity. Western blot analysis showed increased copper transporter ATP7A level and decreased thymidylate synthase level in S3 cells compared with TSGH cells, but the levels of ATP7B were identical. Cellular CDDP accumulation was significantly decreased in S3 cells, whereas oxaliplatin accumulation was similar for both lines. Amounts of oxaliplatin-DNA and CDDP-DNA adducts in S3 cells were about 15% and 40% of levels seen with TSGH cells, respectively. Resistance indexes between S3 and TSGH cells to oxaliplatin and CDDP were both reduced by approximately half when cells were pre-treated with P-type ATPase-inhibitor sodium orthovanadate. Despite elevated glutathione levels in S3 cells, there was no alteration of resistant phenotype to oxaliplatin or CDDP as measured by clonogenic assay when cells were co-treated with L-buthionine-(S,R)-sulfoximine. Host reactivation assay revealed enhanced repair of oxaliplatin-damaged and CDDP-damaged DNA in S3 cells compared with TSGH cells.
**Conclusions:** Together, our results show that the mechanism responsible for oxaliplatin and CDDP resistance in S3 cells is the combination of increased DNA repair and overexpression of ATP7A. Downregulation of thymidylate synthase in S3 cells renders them more susceptible to 5-fluorouracil-induced cytotoxicity.

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**Acquired resistance to oxaliplatin in colon cancer cell lines is associated with up-regulation of G2/M checkpoint**

I. Vasilevskaya, P.J. O'Dwyer. *University of Pennsylvania, Medicine, Philadelphia, USA*

Oxaliplatin has a major role in the treatment of colorectal cancer, and a greater understanding of determinants of sensitivity and resistance is needed. To investigate the molecular basis of acquired resistance, we isolated oxaliplatin-resistant HCT116 and HCT116p53$^{-/-}$ colon cancer sub-lines (HCT.ORC21 and HCTp.ORC17, respectively) by repeatedly exposing parental cells to gradually increasing concentrations of the drug. The $IC_{50}$ concentrations of oxaliplatin established in MTT assays are 25 mkM for HCT.ORC21 and 35 mkM for HCTp.ORC17, demonstrating a significant increase above those of parental HCT116 and HCT116p53$^{-/-}$ cells: 35- and 7-folds, respectively. A significant decrease in the cytotoxicity of oxaliplatin to the resistant cell lines was also observed in colony-forming assays. The resistant cell lines did not exhibit cross-resistance to TRAIL, and demonstrated on average only a 2-fold increase in resistance to cisplatin and 5-fluorouracil, suggesting that impairment of classical apoptotic pathways likely was not the major cause.
Assessment of p53 function in HCT.ORC21 cells suggested the selection for cells with impaired p53 function as one of the mechanisms of acquired oxaliplatin resistance: elevated levels of p53 protein and of its phosphorylation in response to oxaliplatin, loss of p21 and mdm2 induction, and the deregulation of G1 arrest in HCT.ORC21 cells, were all consistent with p53 mutation.
The much lower proliferation rate of both oxaliplatin-resistant cell lines prompted us to evaluate activation of checkpoints and cell cycle responses to oxaliplatin in the whole panel. In HCT116 cell line both expression and activation of Chk1 were abrogated by 48 hours of oxaliplatin treatment, whereas cell lines with impaired p53 demonstrated persistent expression and activation of Chk1 in response to prolonged drug exposure. HCTp.ORC17 cells demonstrated higher sensitivity to Chk1 inhibitor SB218078, both as a single agent and in combination with oxaliplatin, in colony-forming assays than HCT.ORC21 cells, suggesting a critical role of Chk1 in p53 negative genetic background. Also, oxaliplatin-resistant cell lines showed higher continuous expression of cyclin B, as compared to parental cell lines, pointing to profound G2/M arrest upon drug treatment. Our data point to persistent activation of Chk1 and G2/M block in response to drug as characteristics of cells with acquired resistance to oxaliplatin.