185

ZD1839 ('Iressa') improves the antitumour activity of tamoxifen ('Nolvadex') and ICI 182, 780 ('Faslodex') in antihormone responsive breast cancer

J.M.W. Gee[1], M.E. Harper[1], I.R. Hutcheson[1], T.A. Madden[1], D. Barrow[1], J.M. Knowlden[1], R.A. McClelland[1], N. Jordan[1], A.E. Wakeling[2], R.I. Nicholson[1]. [1]*Cardiff University, Tenovus Cancer Research Centre, Cardiff, United Kingdom;* [2]*AstraZeneca, Macclesfield, United Kingdom*

While many oestrogen receptor (ER)-positive breast cancer patients initially respond to antihormonal agents such as tamoxifen ('Nolvadex'), resistance may develop. We have previously demonstrated that the selective epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) ZD1839 ('Iressa') is a potent inhibitor of the elevated EGFR activity, which is essential for proliferation of breast cancer sub-lines that have acquired antihormonal resistance. In contrast, EGFR is barely detectable in the antihormone responsive parental cells (MCF-7) and ZD1839 has little effect on cell growth. In anticipation of the increased dependence on EGFR signalling with development of antihormonal resistance, the present *in vitro* study investigated whether co-treating breast cancer cells with tamoxifen (1x10-7 M) or ICI 182, 780 ('Faslodex') (1x10-7 M) and ZD1839 (1x10-6 M) can promote additional growth inhibitory activity compared with antihormone alone. Tamoxifen decreased cell growth compared with untreated MCF-7 cells, with MIB1 immunostaining and FACS analysis revealing decreased proliferative activity and a reduced S-phase fraction, respectively. Tamoxifen modestly enhanced apoptosis (as measured by annexin V-FITC binding and the ApoAlertTM Mitochondrial Membrane Sensor Kit), with some decreases in the cell survival protein Bcl-2. Co-treatment of MCF-7 cells with tamoxifen plus ZD1839 enhanced anti-proliferative and pro-apoptotic activity compared with tamoxifen alone, while ZD1839 alone had no effect. ICI 182, 780 plus ZD1839 was similarly superior to ICI 182, 780 alone. Studies in ER-positive T47D breast cancer cells confirmed that co-treatment increased inhibitory activity. In parallel with improved growth inhibition, ZD1839 plus tamoxifen blocked antihormone-induced increases in EGFR protein expression, with greater depletion of activated ERK 1/2 MAP kinase (pMAPK) and AKT. These studies demonstrate that ZD1839 in combination with antihormonal agents provides superior antitumour activity in antihormone-responsive breast cancer cells *in vitro*. Indeed, combination treatment may prevent antihormonal resistance, since the obvious outgrowth of EGFR and pMAPK positive cells after 5 weeks' tamoxifen treatment was absent in cultures treated with tamoxifen plus ZD1839. Clinical studies using such combinations of antihormonal agents and EGFR-TKIs are ongoing. 'Iressa', 'Nolvadex' & 'Faslodex' are trademarks of the AstraZeneca group of companies

186

In vitro studies evaluating the interaction between ZD1839 ('Iressa') and ionizing radiation

N. Giocanti[1], C. Hennequin[2], R. Defrance[3], V. Favaudon[1]. [1]*Institut Curie-Recherche, U 350 INSERM, Orsay, France;* [2]*Hôpital Saint-Louis, Service de Cancérologie-Radiothérapie, Paris, France;* [3]*AstraZeneca, Rueil-Malmaison, France*

Introduction: Tumor repopulation during treatment is a major problem in radiation therapy and may be the cause of treatment failure. Over the past decade, treatments combining cytotoxic antitumor drugs and radiation have been developed to take advantage of the synergistic interaction between these modalities. Here we present results from *in vitro* studies to determine whether the cytostatic agent ZD1839 ('Iressa'), an orally active, selective epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), interacts with radiation treatment.
Methods: Three epithelial cancer cell lines with different levels of EGFR expression were used (A431, vulvar carcinoma, EGFR++; A549, lung carcinoma, EGFR±; HeLa, cervix carcinoma, EGFR+). The effect of ZD1839 was investigated as a function of both the concentration (up to 30 μM) and the length of drug exposure (up to 6 days). Irradiation of cells was performed with a 137Cs irradiator. Growth inhibition and cytotoxicity were measured by cell count and colony-forming assays, respectively. DNA double-strand breaks (DSB) were determined by neutral filter elution, and cell cycle progression was measured by flow cytometric analysis with bromodeoxyuridine labeling.
Results: ZD1839 alone reversibly blocked cell proliferation in the three cell lines of interest. The drug consistently elicited S-phase depletion and G1 phase accumulation at concentrations in excess of the IC_{50} determined from cell growth assays (A431, 0.3 μM; A549, 6 μM; HeLa, 8 μM, 48-h contact). No cytotoxicity was observed for drug alone, except in HeLa cells. ZD1839 did not affect the cells' response to ionizing radiation, even after prolonged contact (up to 60 h prior to radiation) with $1xIC_{50}$ of ZD1839. Furthermore, ZD1839 did not affect the incidence and repair of radiation-induced DSB.
Conclusion: The data show that ZD1839 does not impair the rejoining of radiation-induced DNA DSB, and does not alter cell survival following radiation. This suggests that it could be useful to combine ZD1839 with radiotherapy, concomitantly or in close temporal proximity, as it should provide strictly additive interaction without the local and systemic adverse effects inherent in treatments with radiation sensitizers (Balosso J et al, Bull Cancer Radiother 1995; 82: 101-112). 'Iressa' is a trademark of the AstraZeneca group of companies

187

Aspirin induces b-catenin phosphorylation and reduces expression of Akt/PKB

S. Dihlmann, M. Kloor, S. Klein, M. von Knebel Doeberitz. *University of Heidelberg, Inst. of Pathology, Molecular Pathology, Heidelberg, Germany*

Constitutive activation of signal transduction pathways like the Akt/PKB or the b-catenin-pathway play a central role in carcinogenesis. We have previously shown that the proapoptotic non-steroidal anti-inflammatory drugs (NSAIDs) aspirin and indomethacin downregulate b-catenin/TCF-signaling. Upon phosphorylation by glycogen synthase kinase 3-b (GSK3-b), b-catenin is suggested to be targeted for ubiquitin-depentent degradation. To elucidate whether the reduced signaling activity of b-catenin in response to NSAIDs was a result of its enhanced phosphorylation we analyzed colorectal cancer cell lysates by phosphorylation-specific antibodies. In SW948 and SW480 colorectal cancer cells, expressing high levels of b-catenin, the phosphorylation of S33, S37, T41 and S45 time dependently increased in response to aspirin and indomethacin. In contrast, in 293 cells, containing low amounts of b-catenin, NSAID treatment did not result in b-catenin phosphorylation. In unstimulated, resting cells, GSK3 is a constitutively active enzyme that is negatively regulated i.e. by the Wnt pathway and by Akt-induced phosphorylation. Phosphorylation of GSK3-b by Akt results in its inactivation and subsequent accumulation of unphosphorylated b-catenin in several cell types. We therefore analyzed whether the aspirin induced b-catenin phosphorylation might be linked to the Akt/PKB survival signaling pathway. We demonstrate that NSAIDs differentially modulated the phosphorylation pattern of GSK3-b and Akt/PKB. Furthermore, expression of Akt/PKB was reduced upon aspirin treatment. The data undeline the importance of the Akt/PKB pathway as a central player in regulation of cell survival and apoptosis and define it as an important target for anti-cancer therapeutics.

188

Mechanism of 17-B-estradiol-induced ERK1/2 activation in breast cancer cells: a role for HER2 and PKC-delta

K. Reddy, V. Keshamouni. *Wayne State University, Pathology, Detroit, USA*

Activation of mitogen-activated protein kinase (Erk/MAPK) is a critical signal transduction event for estrogen (E2)-mediated cell proliferation. Recent studies from our group and others have shown that persistent activation of Erk plays a major role in cell migration and tumor progression. The signaling mechanism(s) responsible for persistent Erk activation are not fully characterized, however. In this study, we have shown that E2 induces a slow but persistent activation of Erk in MCF-7 breast carcinoma cells. The E2-induced Erk activation is dependent on new protein synthesis, suggesting that E2-induced growth factors play a major role in Erk activation. When MCF-7 cells were treated with E2 in the presence of an anti-HER-2 monoclonal antibody (Herceptin), 60 -70% of E2-induced Erk activation is blocked. In addition, when untreated MCF-7 cells were exposed to conditioned medium (CM) from E2-treated cells, Erk activity was significantly enhanced. Furthermore Erk activity was blocked by an antibody against HER-2 or by heregulin (HRG) depletion from the conditioned medium through immunoprecipitation. In contrast, epidermal growth factor receptor (EGFR) (Ab 528) antibody only blocked 10-20% of E2-induced Erk activation, suggesting that E2-induced Erk activation is predominantly mediated through the secretion of HRG and activation of HER-2 by an autoctine/paracrine mechanism. Inhibition of PKC-d mediated signaling by a dominant negative mutant or the relatively specific PKC-d inhibitor rottlerin blocked most of the E2-induced Erk activation, but had no effect on TGFa-induced Erk activation. By contrast, inhibition of Ras, by inhibition of farnesyl transferase (Ftase-1) or dominant negative (N17)-Ras significantly inhibited both E2

and TGFa induced Erk activation. This evaluation of downstream signaling revealed that E2-induced Erk activation is mediated by a HRG/ HER-2/PKC-d/Ras pathway that could be crucial for E2-dependent growth-promoting effects in early stages of tumor progression.

189

17-DMAG (NSC 707545), a water-soluble geldanamycin analog, has superior *in vitro* and *in vivo* antitumor activity compared to the hsp90 inhibitor 17-AAG

V. Smith[1], E.A. Sausville[2], R.F. Camalier[2], H.H. Fiebig[1], A.M. Burger[1]. [1]*Tumor Biology Center at the University of Freiburg, Freiburg, Germany;* [2]*National Cancer Institute, DTP, DCTD, Bethesda, USA*

The heat shock protein 90 (hsp90) is involved in the correct folding of several signal transduction kinases such as erbB2, PI3K and raf-1. 17-allylamino-geldanamycin (17-AAG) is an inhibitor of hsp90 and currently in clinical trials. However, difficulties in formulation have lead to the development of the water-soluble 17-(dimethylaminoethylamino-17- demethoxygeldanamycin (17-DMAG). In the present study, the two compounds were compared to contrast their behavior. We had found previously that human melanoma cells were responsive to 17-AAG. Therefore this tumor type was investigated. Growth inhibition (IC_{50}, IC100) was assessed in the melanoma cell lines MEXF 276L, MEXF 462NL and MEXF 514L using a 96 hr SRB assay. 17-DMAG was more potent than 17-AAG with an IC_{50} in the sensitive MEXF 276L of 37 nM for 17-DMAG and 187 nM for 17-AAG. MEXF 514L was resistant to both compounds (IC_{50}>8 μM). Additionally, clonogenic assays were performed on a panel of 13 human tumor xenografts. The mean IC_{50} for inhibition of colony formation was lower for 17-DMAG than for 17-AAG (20 nM vs. 39 nM). These results translated into *in vivo* activity. In 2/3 s.c. growing melanoma xenografts both compounds were active at their MTD, but e.g. the growth delay in mice bearing MEXF 276 tumors was 16 d for 17-DMAG (15 mg/kg given 2x QdX5 i.v. in PBS) and only 11 d for 17-AAG (60 mg/kg, 2x Qdx5 i.p. as a DMSO/PBS suspension). MEXF 514 xenografts however, did not respond. The most marked difference between the sensitive and resistant melanomas is the expression of erbB2. The latter is prominently expressed in the MEXF 276L model but undetectable in MEXF 514L cells. In order to compare the behavior of the agents on a molecular basis, the modulation of hsp90 and its client proteins were assessed via immunoblotting after exposure to IC100. Here, 17-DMAG and 17-AAG were identical: hsp90 protein levels decreased in MEXF 276L whilst in MEXF 514L cells the expression did not change; c-raf-1 protein was reduced in MEXF 276L cells, but not in MEXF 514L. No change in protein expression was observed for PI3K. MEXF 276L showed a decrease in erbB2 protein levels concomitantly with loss of hsp90. Our data demonstrates that while both compounds inhibit signal transduction through hsp90 modulation, the efficacy and pharmaceutical properties for 17-DMAG are superior to those of 17-AAG. 17-DMAG should be regarded as therefore having potential advantages for clinical development in comparison to 17-AAG.

190

The role of G1 and G2 checkpoint control proteins involved in cell cycle arrest following treatment with the HSP90 inhibitor 17AAG

A. Maloney, M. Walton, J. Titley, P. Workman. *Institute of Cancer Research, Cancer Research UK Centre for Cancer Therapeutics, Sutton, United Kingdom*

The HSP90 inhibitor 17-allylamino, 17-demethoxygeldanamycin (17AAG) exerts its antitumour effect by inhibiting the intrinsic ATPase activity of the molecular chaperone HSP90. This causes the depletion of key oncogenic proteins via the ubiquitin proteosome pathway, resulting in both cytostasis and apoptosis. However, the effects of 17AAG on cell cycle checkpoint kinase expression have not been explored in any great detail and may be crucial determinants in cell cycle control. In this study, cell cycle kinetics were examined in the A2780, HT29 and Lovo tumour cell lines by continuously labelling cells with bromodeoxyuridine and performing bivariate Hoechst/PI flow cytometric analysis. The expression of a number of key cell cycle checkpoint proteins and upstream signalling proteins were examined using Western blotting and RNase Protection assays (RPA). In the A2780 cell line (p53+/+) cells from all phases of the cell cycle accumulated predominantly in the G1 phase of the cell cycle. In the Lovo (p53+/+) and HT29 (p53-/-) cell lines, G1 and G2 cells were blocked in the cell cycle phases in which they originated, with S phase cells accumulating in G2/M. However, the G2/M arrest was leaky in the HT29 cells and by 16h some cells progress through to the G1 phase of the cell cycle, suggesting differential regulation of the G2 checkpoint by 17AAG in these cell lines. p53 and p21 induction was observed at 24hr in cells expressing wild type p53 whereas mutant p53 was depleted in HT29 cells with no evidence of p21 induction. A decrease in RB phosphorylation in A2780 and HT29 cells was observed consistent with the observed G1/S arrest. However, in Lovo cells there was no obvious phospho RB signal suggesting RB function may be compromised. Following 17AAG treatment, protein expression levels of a number of kinases involved in cell cycle checkpoint control were depleted including CDK4, WEE1 and CHK1. RPA data indicated no drug induced changes in mRNA expression of these kinases, suggesting they are not transcriptionally regulated by 17AAG. To conclude, the G1 or G2 arrests observed with 17AAG in these cell lines are not simply related to the p53 or RB status of the cell line, although these pathways may play a crucial role in the maintenance of the cell cycle response. Depletion of key cell cycle checkpoint kinases may be an important factor in determining cell cycle response following 17AAG treatment.

191

Additive interaction of platinum compounds and 17-AAG in colon cancer cell lines depends on intact JNK signaling

I. Vasilevskaya, T. Rakitina, P. O'Dwyer. *University of Pennsylvania, Medicine, Philadelphia, USA*

We showed earlier that in the HT29 colon adenocarcinoma cell line 17-AAG antagonizes the action of cisplatin, while exerting additive effects in HCT116 cells. Since the importance of p53 status and integrity of stress signaling pathways in cellular responses to platinum compounds is well established, we investigated if the possible interference by 17-AAG with cisplatin-induced signaling and apoptosis is one of the reasons for their antagonism. To evaluate the role of signaling pathways in the interaction, we studied four colon cancer cell lines with different p53 status: HCT116, with intact p53, and HT29, DLD1 and SW480 cell lines, bearing p53 mutations. Clonogenic assays demonstrated higher sensitivity to 17-AAG and platinum agents in HCT116 and DLD1 cells, compared to those of HT29 and SW480. To assess the effect of combined treatment on signaling through MAPK cascades, cells were treated for 24 hours with 3xIC90 concentrations of each drug alone and in combination. In HCT116 and DLD1 cell lines c-Jun induction by cisplatin was somewhat inhibited by 17-AAG, whereas in HT29 and SW480 cells it was completely abrogated. Further, in HT29 cells the MAPK and JNK signaling pathways were strongly inhibited when cells were exposed to cisplatin in the presence 17-AAG. Treated in the same manner, SW480 cells demonstrated the loss of JNK activation and inhibition of ATF2 and c-Jun phosphorylation, while p38 activation was unaffected. In HCT116 and DLD1 cell lines all major signaling pathways were intact, demonstrating only partial overall inhibition. In addition to disruption of cisplatin-induced JNK pathway activation, 17-AAG treatment led to inhibition of both basal and cisplatin-induced caspase 8 activity in HT29 cells while in the HCT116 cell line it was unaffected. These data suggest that an additive response to combined platinum drug with 17-AAG depends on intact apoptotic signaling, especially through JNK. The data emphasize the care required in combining a stress signal inducer (cisplatin) with a signaling inhibitor (17-AAG).

192

Activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor (VEGFR [KDR]-TKI), in a model of ZD1839 ('Iressa') resistance

F. Taguchi[1,3], Y. Koh[1], F. Koizumi[1], Y. Ueda[1], S. Tsukiyama[1], T. Tamura[2], N. Saijo[2], K. Nishio[1]. [1]*National Cancer Center Research Institute, Pharmacology, Tokyo, Japan;* [2]*National Cancer Center Hospital, Medical Oncology, Tokyo, Japan;* [3]*Tokyo Medical University, Surgery, Tokyo, Japan*

ZD6474 is a novel inhibitor of vascular endothelial growth factor receptor (VEGFR [KDR]) signaling that inhibits angiogenesis and tumor growth in a range of tumor models. In addition, ZD6474 has some activity against EGFR tyrosine kinase. ZD1839 ('Iressa') is an orally active, selective epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) that blocks signal transduction pathways implicated in the proliferation and survival of cancer cells. We have established a human lung cancer cell line that is resistant to ZD1839 (PC-9/ZD) and have now investigated the direct tumor inhibitory activity of ZD6474. In an MTT proliferation assay, ZD6474 showed partial cross-resistance to PC-9/ZD cells suggesting that EGFR-inhibitory activity partially contributes to the growth-inhibitory effect of this compound on tumor cells in culture. To elucidate the effects of ZD6474