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Simultaneous targeting of tumor cell proliferation and tumor-induced neoangiogenesis by the novel CDK2/VEGF-RTK dual pathway inhibitor ZK-CDK

G. Siemeister, H. Briem, T. Brumby, M. Haberey, R. Jautelat, M. Krüger, U. Lücking, A. Reichel, M. Schäfer, K. Bosslet. *Schering AG, Corporate Research, Berlin, Germany*

Loss of cell cycle control and tumor induced neovascularization represent two major hallmarks of cancer. Cyclin-dependent kinases (CDKs), a family of Ser/Thr kinases which requires association with a cyclin regulatory subunit for activation, are required for the correct timing and order of the events of the cell divison cycle. Aberrant CDK control and consequent loss of cell cycle checkpoint function have been directly linked to the molecular pathology of cancer. Tumor induced neoangiogenesis was identified as a crucial mechanism to achieve blood supply for tumor growth, invasion and metastasis. The endothelial cell specific vascular endothelial growth factor (VEGF)/VEGF-receptor tyrosine kinase (VEGF-RTK) system has been validated as a main signaling pathway in tumor angiogenesis. In addition, the related platelet-derived growth factor (PDGF)-RTKs contribute to tumor angiogenesis.
Using a human tumor xenograft model we have observed synergistic efficacy of a combination treatment consisting of a CDK mono-pathway inhibitor and a pan-VEGF-/PDGF-RTK inhibitor. Based on this finding we have developed highly potent inhibitors with dual pathway specificity for CDKs and VEGF-/PDGF-RTKs from a program to identify low molecular weight CDK2 inhibitors. In particular, the selected compound for clinical development (status: phase I) ZK-CDK inhibited CDK2/cyclinE, CDK1/cyclinB, VEGF-RTK1–3 and PDGF-RTKß in the low nanomolar range, and inhibited the proliferation of human tumor cell lines with a mean IC50 of 250 nM. ZK-CDK blocked cell cycle progression in G1 and induced apoptosis. ZK-CDK blocked VEGF-induced vascular permeability in vivo and reduced blood supply of human tumor xenografts. Upon oral dosing of ZK-CDK significant tumor growth inhibition was observed in a variety of human tumor xenograft models. ZK-CDK was particulary efficacious in slowly growing, hormone-independent, p53-negative, and multidrug-resistant tumors. Daily oral application of ZK-CDK at therapeutic doses was well tolerated by nude mice over a periode of time of more than 70 days.
The dual mechanism of action of ZK-CDK attacking tumor cells via inhibition of cell cycle progression and, simultaneously, attacking tumor neovascularization via inhibition of VEGF- and PDGF-RTKs results in highly efficacious inhibition of growth of human tumor xenografts.

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The ATM-Chk2 kinase pathway: molecular interaction maps and therapeutic rationale

Y. Pommier, M. Aladjem, K.W. Kohn. *National Cancer Institute, Laboratory of Molecular Pharmacology, Bethesda, USA*

Most anticancer agents presently used clinically target the genome by damaging DNA. Their tumor selectivity is probably due to tumor-specific defects affecting cell cycle checkpoints and DNA repair, and enhancing apoptotic response in the tumor. To help elucidate which tumors will be candidates for specific therapies, we use the molecular interaction map convention (MIM) (1–4) to organize and analyze molecular networks involved in cell cycle regulation. Here we show a MIM representing the ATM-Chk2 pathway. This pathway includes the DNA damage sensor kinases (ATM, ATR and DNA-PK), the adaptor BRCT proteins (Nbs1, Brca1, 53BP1, MDC1) and the effector kinases (Chk2, Chk1, Plk3, JNK, p38). A characteristic of the ATM-Chk2 pathway is its apparent redundancy. For instance, ATM and Chk2 have common substrates including p53, PML, E2F1, BRCA1, and Chk2 itself, suggesting that Chk2 (also known as CHECK2, Cds1 in fission yeast, and Dmchk2 or Dmnk or Loki in the fruit fly) can act as a relay for ATM and/or as a salvage pathway when ATM is inactivated. Some of the same substrates can also be phosphorylated/activated (even at similar residues) by other pathways than ATM-Chk2, including Chk1, and the polo kinases (Plk's). This map can be accessed on the Web, along with interactive plug-ins, and links (4). Functionally, Chk2 can activate both apoptosis (via p53, and PML) and cell cycle arrest at S-phase (via cdc25A and cdc25C, p53, and BRCA1). We will discuss the rationale for using Chk2 inhibitors to enhance the tumor selectivity of DNA damaging agents in p53-deficient tumors, and for the treatment of tumors with enhanced Chk2 activation. 1. Kohn, K. W. Molecular interaction map of the mammalian cell cycle control and DNA repair systems. Mol Biol Cell, 10: 2703–2734, 1999. 2. Pommier, Y., Sordet, O., Antony, S., Hayward, R. L., and Kohn, K. W. Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. Oncogene, 23: 2934–2949, 2004. 3. Aladjem, M. I., Pasa, S., Parodi, S., Weinstein, J. N., Pommier, Y., and Kohn, K. W. Molecular interaction maps--a diagrammatic graphical language for bioregulatory networks. Science STKE, 2004: pe8, 2004. 4. http://discover.nci.nih.gov/mim.

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Cytoplasmic localization of p21 cip1/WAF1 is a poor prognostic marker in the breast cancer patients

W. Xia[1], J.-S. Chen[1], X. Zhou[2], D.-F. Lee[1], Y. Liao[1], B. Zhou[1], M.-C. Hung[1]. *[1]UT M.D. Anderson Cancer Center, Molecular & Cellular Oncology, Houston, USA; [2]UT M.D. Anderson Cancer, Biostatistical, Houston, USA*

Purpose: The diversity of biological functions marker p21 $^{Cip1/WAF1}$ (p21) is a controversial marker in predicting the prognosis of breast cancer patients. Recent laboratory studies have revealed that the regulation of p21 function could be related to the subcellular localization of p21 by Akt-induced phosphorylation at threonine 145 in HER2/neu-overexpression breast cancer cells. The purpose of this study was to verify these findings in clinical settings.
Experimental Design: The expression status of key biological markers in the HER2/neu-Akt-p21 pathway in 130 breast cancer specimens was evaluated using immunohistochemical staining and correlated with patients' clinical parameters and survival. In addition, an antibody against phospho-p21 at threonine 145 [phospho-p21 (T145)] was also used for better validation of these findings.
Results: Cytoplasmic localization of p21 was found to be highly correlated with overexpression of phosphore-p21 (T145). Both cytoplasmic p21 and overexpression of phospho-p21 (T145) were associated with high expression levels of HER2/neu and phospho-Akt. Cytoplasmic localization of p21 as well as overexpression of phospho-p21 (T145), HER2/neu, and phospho-Akt were all associated with poor overall survival. Multivariate analysis of the Cox proportional hazard regression model revealed that cytoplasmic p21 and overexpression of HER2/neu are independently associated with increased risk of death. Combining these two factors stratifies patients' survival into four distinct groups, with a 5-year survival rate of 79% in low HER2/neu and negative/nuclear p21 patients, 60% in high HER2/neu and negative/nuclear p21 patients, 29% in low HER2/neu and cytoplasmic p21 patients, and 16% in high HER2/neu and cytoplasmic p21 patients.
Conclusions: The present study supports the mechanisms of p21 regulation derived from the earlier laboratory investigation, demonstrates the prognostic importance of phospho-p21 (T145) for the first time, and also provides a novel combination of p21 and HER2/neu for better stratification of patients' survival than any single clinicopathological or biological marker, which may play important diagnostic and therapeutic roles for breast cancer patients.

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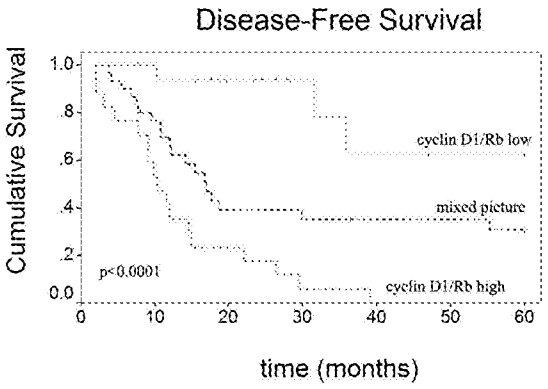
Automated quantitative in-situ method of protein analysis (AQUA) demonstrates that components of the retinoblastoma (Rb) pathway are potential molecular targets in oropharyngeal squamous cell cancer

A. Psyrri[1], Y. Ziwei[2], P.M. Weinberger[2], B. Haffty[3], C. Sasaki[2], R.L. Camp[4], D.L. Rimm[4]. *[1]Yale University, Medical Oncology, New Haven, CT, USA; [2]Yale University, Otolaryngology, New Haven, CT, USA; [3]Yale University, Therapeutic Radiology, New Haven, CT, USA; [4]Yale University, Pathology, New Haven, CT, USA*

Background: The current TNM system is inadequate to accurately classify patients in terms of prognosis. Thus, considerable interest lies in discovering molecular prognostic factors using proteomic technologies. Our aim was to investigate the correlation of expression levels of cell cycle regulatory proteins cyclin D1, pRb and p16 with patient prognosis in a cohort of patients with oropharyngeal squamous cell carcinoma.
Methods: We studied the protein expression levels of cyclin D1, pRb and p16 on a tissue microarray composed of 109 oropharyngeal squamous cell carcinomas with long-term follow-up data available. Protein expression was analyzed with an automated in-*situ* quantitative method (AQUA) which allows preservation of tissue morphology while quantifying protein expression in paraffin embedded tissue.
Results: The mean follow-up time was 36 months. Patients with high expression levels of the cyclin-dependent kinase (CDK) inhibitor p16 had decreased local recurrence and improved disease-free survival. Patients with elevated levels of Rb expression had increased local recurrence and worse disease-free survival, as did patients with elevated cyclin D1 expression ($p<0.01$ for each). Patients with high p16 expression had lower Rb expression ($p=0.001$) but no difference in cyclin D1 expression. In multivariate Cox regression, cyclin D1 and Rb expression levels were independent predictors of disease-free survival and local recurrence. When cyclin D1 and Rb expression patterns were combined, tumors could be

classified into 3 risk strata (high cyclin D1 and Rb, low cyclin D1 and Rb, and mixed). Tumors in the high-risk group had a 5-year disease free survival of 0% versus 63% in the low risk group (p<0.0001).



Conclusion: Our results demonstrate the power of AQUA in identifying molecular targets for cancer therapy. The association of low levels of p16 and high levels of cyclin D1 with poor survival indicates that CDK inhibitors may have a role in treatment of patients with oropharyngeal tumors. Additionally, the association of elevated p16 levels with low pRb expression may indicate a causal role of Human Papillomavirus (HPV) in this tumor subset. It is known that oncoprotein E7 of high-risk HPVs binds and degrades Rb protein. Experiments in our laboratory are underway to explore the association between HPV positivity and pRb /p16 levels. If this holds true, then HPV targeted therapies can be used in oropharyngeal tumors with p16 overexpression.

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The combination of Indisulam (E7070) with cisplatin, oxaliplatin and 5-fluorouracil are synergistic in vitro and in vivo

Y. Ozawa, T. Owa, A. Yokoi, K. Yoshimatsu, M. Asada. *Eisai Co., Ltd., Tsukuba Research Laboratories, Tsukuba-shi, Japan*

Indisulam is an experimental anticancer agent that is presently completing phase II studies in solid tumours. DNA array analysis indicates that indisulam down-regulates several genes that are related to cytotoxic drug resistance, including cyclin H and general transcription factor IIH 62kDa-subunit (GTF2H1) [involved in nucleotide excision repair], glutathione synthetase (GS) [related to detoxification of platinum], thymidylate synthetase (TS) [target molecule of 5-fluorouracil (5-FU)], and so on. To determine the importance of these events in establishing combination therapies with indisulam, we investigated the efficacy of indisulam in combination with cisplatin, carboplatin, oxaliplatin and 5-FU *in vitro* and *in vivo*. *In vitro* studies were performed using HCT15 colorectal cancer cells. Combination effects were analyzed by MTT assay and isobologram analysis. Initial exposure to indisulam followed by cisplatin, carboplatin, oxaliplatin or 5-FU were synergistic. *In vivo* studies were performed using an HCT15 xenograft model (cisplatin and 5-FU), a Lu99 (NSCLC) xenograft model (cisplatin) and a Colo320D. M.(colorectal) xenograft model (5-FU). In the HCT15 xenograft model (simultaneous administration), indisulam + CDDP and indisulam + 5-FU demonstrated tendency of synergy, even as CDDP alone and 5-FU alone showed no effect. In Colo320D. M. xenograft model, indisulam + 5-FU had tendency of synergy in the indisulam pre-administration schedule, but not in other administration schedules. In the Lu99 xenograft model, indisulam + cisplatin also showed synergistic effects only in the indisulam pre-administration schedule. The present data suggest that the optimal combination schedule in combination of indisulam with platinum-containing drugs or 5-FU is the administration of indisulam preceding platinum or 5-FU. The synergistic efficacy of Indisulam with platinum and 5-FU in the schedule may be attributable to increasing the chemosensitivity to platinum-containing drugs and 5-FU, at least partially, due to cyclin H, GTF2H1, GS and TS suppression caused by Indisulam. From these data, clinical trials of indisulam in combination with carboplatin, oxaliplatin and 5-FU are ongoing.

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Repression of cell cycle-related proteins by oxaliplatin but not cisplatin in human colon cancer cells

C. Voland, A. Bord, A. Peleraux, G. Penarier, D. Carriere, O. Jbilo, P. Casellas. *Sanofi-Synthelabo Recherche, Immunology – Oncology, Montpellier, France*

Oxaliplatin (*cis*-[(1*R*,2*R*)-1,2-cyclohexanediamine-*N, N'*] oxalato (2-)-*O, O'* platinum; Eloxatin) is a third-generation platinum which has an *in vitro* and *in vivo* spectrum of activity distinct from that of cisplatin, especially in colon cancer cells. Here, we studied the molecular basis of this difference on the human colon carcinoma HCT-116 cell line. Oxaliplatin inhibited HCT-116 cell proliferation with greater efficacy than cisplatin (IC_{50} at 0.4 μM and 1.2 μM for oxaliplatin and cisplatin, respectively). At equicytotoxic concentration, oxaliplatin activated the G1 and G2/M checkpoints while cisplatin activated the S and G2/M checkpoints. To search for oxaliplatin-specific target genes and mechanism different from those of cisplatin, we established the transcriptional signatures of both products on HCT-116 cells using the microarray technology and found from hierarchical clustering that i) much more genes were modulated by oxaliplatin compared with cisplatin, ii) among the 117 modulated genes, 79 genes were similarly regulated by both drugs and in sharp contrast, 38 genes were dose-dependently down-regulated by oxaliplatin and conversely up-regulated or unaffected by cisplatin. Interestingly among these latter group is a number of cell cycle-related genes, encoding proteins involved in DNA replication (CDC6, primase, cyclin A, replication protein A and replication factor C), and G2/M cell cycle progression (cyclin B1, Cdk1, and CDC25C). RNA modulations, confirmed at the protein level, were in accordance with the oxaliplatin effects on the cell cycle. Furthermore, given the modulations of cyclins and cdks, we examined the nucleolus using an electron microscopic approach and evidenced that oxaliplatin dramatically affected the nucleolar architecture. Interestingly cisplatin, which did not repress these genes, had no effect.
Beyond the identification of target genes that are affected by both drugs, the identified oxaliplatin-specific target genes could be additionally useful as predictive markers to evaluate and compare the efficacy of platinum drugs.

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The mode of action of CYC202 (R-roscovitine) a cyclin dependent kinase inhibitor currently in phase II clinical trials

S. Green, D.E. MacCallum, I.N. Fleming, J. Melville, M. Watson, S. Frame, K. Watt, S. Anderson, A. Gianella-Borradori, D.P. Lane. *Cyclacel Ltd, Dundee, UK*

CYC202 is a potent Cyclin Dependent Kinase (CDK) inhibitor, competing with ATP for its binding site on the kinase. It has greatest in vitro activity against CDK2/Cyclin E, CDK7/Cyclin H and CDK9/Cyclin T. CYC202 inhibits both cell cycle progression and transcription by RNA polymerase II. CYC202 induces apoptosis from all phases of the cell cycle in tumour cell lines and reduces tumour growth in human tumour xenografts in nude mice. Two Phase I studies have been completed and three Phase II trials are ongoing: in combination with gemcitabine/cisplatin for NSCLC; in combination with capecitabine for breast cancer; and as a single agent in haematological B-cell malignancies
In order to identify potential biomarkers that may be useful for clinical development of CYC202, cellular mode-of-action experiments have been performed in both multiple myeloma and solid tumour cell lines. In cell lines from both tumour types, CYC202 induced rapid dephosphorylation of the retinoblastoma protein and the C-terminal domain (CTD) of RNA polymerase II. Phosphorylation of RNA polymerase II is crucial for its role in transcription and is controlled by CDKs 2, 7, 8 & 9 while Rb is phosphorylated by CDKs 2 & 4; thus in cells, CYC202 appears to be inhibiting several different CDKs. Inhibition of CDKs 7 amp; 9 would block transcription and would be predicted to exert its greatest effect on gene products where both mRNA and protein have short half-lives; one such gene product is the anti-apoptotic factor Mcl-1 which inhibits pro-apoptotic proteins and is essential for survival of a range of cell types including multiple myeloma. As hypothesised, treatment of multiple myeloma cells with CYC202 caused rapid down regulation of Mcl-1, this correlated with the induction of apoptosis as determined by TUNEL and PARP cleavage. A significant reduction in Mcl-1 was also seen in solid tumour cell lines.
Results will be presented detailing these ongoing experiments examining the cellular mode-of-action of CYC202.