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Resistance of Cancer Cells to Targeted Therapies Through the Activation of Compensating Signaling Loops

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Abstract: The emergence of low molecular weight kinase inhibitors as "targeted" drugs has led to remarkable advances in the treatment of cancer patients. The clinical benefits of these tumor therapies, however, vary widely in patient populations and with duration of treatment. Intrinsic and acquired resistance against such drugs limits their efficacy. In addition to the well studied mechanisms of resistance based upon drug transport and metabolism, genetic alterations in drug target structures and the activation of compensatory cell signaling have received recent attention. Adaptive responses can be triggered which counteract the initial dependence of tumor cells upon a particular signaling molecule and allow only a transient inhibition of tumor cell growth. These compensating signaling mechanisms are often based upon the relief of repression of regulatory feedback loops. They might involve cell autonomous, intracellular events or they can be mediated *via* the secretion of growth factor receptor ligands into the tumor microenvironment and signal induction in an auto- or paracrine fashion. The transcription factors Stat3 and Stat5 mediate the biological functions of cytokines, interleukins and growth factors and can be considered as endpoints of multiple signaling pathways. In normal cells this activation is transient and the Stat molecules return to their non-phosphorylated state within a short time period. In tumor cells the balance between activating and de-activating signals is disturbed resulting in the persistent activation of Stat3 or Stat5. The constant activation of Stat3 induces the expression of target genes, which cause the proliferation and survival of cancer cells, as well as their migration and invasive behavior. Activating components of the Jak-Stat pathway have been recognized as potentially valuable drug targets and important principles of compensatory signaling circuit induction during targeted drug treatment have been discovered in the context of kinase inhibition studies in HNSCC cells [1]. The treatment of HNSCC with a specific inhibitor of c-Src, initially resulted in reduced Stat3 and Stat5 activation and subsequently an arrest of cell proliferation and increased apoptosis. However, the inhibition of c-Src only caused a persistent inhibition of Stat5, whereas the inhibition of Stat3 was only transient. The activation of Stat3 was restored within a short time period in the presence of the c-Src inhibitor. This process is mediated through the suppression of P-Stat5 activity and the decrease in the expression of the Stat5 dependent target gene SOCS2, a negative regulator of Jak2. Jak2 activity is enhanced upon SOCS2 downregulation and causes the reactivation of Stat3. A similar observation has been made upon inhibition of Bmx, bone marrow kinase x-linked, activated in the murine glioma cell lines Tu-2449 and Tu-9648. Its inhibition resulted in a transient decrease of P-Stat3 and the induction of a compensatory Stat3 activation mechanism, possibly through the relief of negative feedback inhibition and Jak2 activation. These observations indicate that the inhibition of a single tyrosine kinase might not be sufficient to induce lasting therapeutic effects in cancer patients. Compensatory kinases and pathways might become activated and maintain the growth and survival of tumor cells. The definition of these escape pathways and their preemptive inhibition will suggest effective new combination therapies for cancer.

Keywords: Drug resistance, signaling redundancy and compensation, targeted tumor therapy.

1. TARGETED CANCER THERAPY

The somatic mutations found in cancer cells have made important contributions to the understanding of the etiology of the disease. 20 distinct mutational signatures have been identified and associations with stages of cancer development could be established. These findings have had important practical implications for the prevention, diagnosis and therapy. Although the total number of mutations found in tumor cells is rather large, only a relatively small fraction of the affected genes contribute to cellular transformation. Most mutations do not consistently occur in a high percentage of the tumors examined. About 140 genes have been designated as "drivers", genes which promote tumorigenesis when they experience changes in their functions through intragenic mutations. Most tumor cells express only a few genes in which functional alterations of "driver genes" have occurred. The large majority of mutations are found in "bystander genes", genes which do not functionally contribute to tumorigenesis. The functions of the "driver genes" can further be defined, they affect 12 signaling pathways and regulate cell fate, cell survival and genome maintenance [2]. These insights into particular mutations and their consequences for signaling pathways and cellular phenotypes, have already had crucial effects on the development of more effective

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drugs and the reduction of cancer morbidity and mortality [3].

Cancer drug discovery has exploited these insights and has focused on the development of therapeutic compounds which interfere with the functions of proteins central to tumorigenesis. The strategies of cancer treatment shifted from nonspecific, cytotoxic drugs to selective drugs, drugs supposed to interfere with defined molecular processes characteristic for particular cancer cells. Although nonspecific, chemotherapeutic agents remain widely used in current cancer treatment, they have a narrow therapeutic index, significant toxicities and tumor cells frequently acquire resistance against these agents. Targeted drugs are designed to inhibit molecular pathways that are crucial for tumor cell survival and growth through interference with the relevant biochemical pathways or essential protein components [4, 5]. Most impressive results have been obtained and tumor growth inhibition or tumor regression have been observed in many patients. The choice of drug is dependent upon the identification of a suited target molecule, usually a mutated version of a "driver gene" product. The principle was originally established in cells of chronic myelogenous leukemia patients. The BCR-ABL kinase inhibitor imatinib induced complete cytogenetic responses in a large fraction of treated patients. Other specific inhibitors for oncoproteins were subsequently derived, e.g. for the EGFR, B-RAF, KIT, HER2 and the anaplastic lymphoma kinase (ALK). Similar to imatinib and its use against chronic myelogenous leukemia, molecularly targeted therapies which inhibit specific pathways essential for cancer development and metastasis have been used in the treatment of colorectal, breast, head and neck, non-small cell lung, renal cell cancers, lung, gastric, cervical, uterine melanoma and brain tumors. They resulted in impressive benefits for selected patient populations and were accompanied by fewer adverse effects [6].

2. RESISTANCE MECHANISMS, CAUSES FOR THE LIMITED DURATION OF THE EFFECTS OF THERAPEUTICS

Chemotherapy has been a principal mode for the treatment of cancer patients over many decades. Chemotherapeutic agents often cause tumor remissions early upon the onset of treatment. In many cases, however, drug resistance develops with time and progressive disease results. The mechanisms of resistance to chemotherapeutic drugs have been studied extensively [7, 8]. Multidrug resistance (MDR) is a condition in which cancer cells are protected from the toxic effects of diverse cancer drugs with different chemical structures and modes of action. Two classes of transporter proteins at the cellular surface are mainly responsible for MDR of tumors. The adenosine triphosphate-binding cassette transporter family is an energy-dependent efflux pump which extrudes toxic chemotherapeutic drugs from the cancer cells. The solute carrier transporter family mediates the cellular uptake of drugs and drug resistance can result from the decreased activity of these transporters. MDR transporters are also expressed in cancer stem cells and provide for their self-protection against chemotherapeutic drugs. The mechanisms of MDR, however, are not limited to reduced drug accumulation, but can also involve changes in the level of target proteins, mutations which diminish drug binding, trapping of drugs in acidic vesicles, enhanced metabolism of drugs by cytochrome P450 mixed function oxidases, increased tolerance of cellular DNA damage and diminished apoptotic signaling [7].

Cancer biology has advanced through the identification of targets in cancer cells which are activated by mutations or alterations in upstream effectors and which promote unregulated cell growth, invasion and metastasis. Cancer treatment has benefited from the discovery and development of agents which target such components. The aim of these therapeutic strategies is the induction of apoptosis, necrosis, senescence or differentiation in cancer cells. The mechanisms to achieve these goals can vary widely, they can be based on cancer cell autonomous processes, ligand secretion and paracrine signaling or heterotypic cell interactions. Preferred targets are proteins which are unconditionally required to establish or maintain the oncogenic state [9, 10]. These strategies are successful and have been widely exploited, mainly through the derivation of specific protein kinase inhibitors [11, 12].

The application of selective kinase inhibitors is guided by the identification of kinases which are deregulated in a particular cancer patient. However, even if the patients respond to the targeted therapy with a specific kinase inhibitor, the phenotypic benefits for the patient, i.e. tumor remission and tumor cell growth inhibition, are not necessarily persistent. The problem of the emergence of drug resistance is not limited to conventional chemotherapeutic drugs, but extends to drugs with a targeted modes of action [13]. Resistance can occur early and manifest itself in the failure of a drug to induce a therapeutic response or resistance can be acquired during the continued administration of the drug. Drug transport and metabolism, similar to the factors influencing resistance towards non-specific chemotherapeutic agents, could play a role.

The reasons for targeted drug resistance need to be further investigated and prospective knowledge about the sensitivity or resistance of a particular tumor to a targeted drug will be of great clinical and economic significance. DNA sequence analyses have been employed to investigate the mechanisms which cause acquired resistance to kinase inhibitors during treatment and which are responsible for relapse of the disease. These efforts have shown that a large fraction of cases of acquired resistance, e.g. to the EGF receptor specific tyrosine kinase inhibitors, can be traced to mutations in the drug target. The mutation T790M within the EGFR kinase domain in tumor cells of non-small cell lung cancer (NSCLC) patients treated with an EGFR specific tyrosine kinase inhibitor, renders the EGFR insensitive to the drug [14]. Similar mutation of 'gatekeeper' residues, from a threonine residue to a larger hydrophobic residue, have been found in other oncogenic kinases, e.g. Bcr-Abl, FLT3 (FMS-like tyrosine kinase 3), PDGFR, FGFR and c-Src [12]. Such kinase domain mutations, which cause a reduction of drug binding and at the same time retain the catalytic function of the enzymes, might explain a large fraction of cases of acquired resistance. The structural and functional consequences of such mutations have been extensively studied in tumor cells from chronic myeloid leukemia patients [15] and non-small cell lung cancer patients [16]. The observation that acquired drug resistance can limit the clinical efficacy of kinase inhibitors led to the development of second

generation compounds. The molecular mechanisms and the structural alterations which caused drug resistance in the first place, especially the discovery of gatekeeper mutations, served as guiding principles [17]. However, all targeted drugs employed for cancer treatment might eventually cause the selection of resistant cell derivatives.

3. EXAMPLES FOR SIGNALING CROSSTALK AND DRUG MEDIATED ACTIVATION OF COMPENSATORY PATHWAYS

Resistance to specific kinase inhibitors usually involves mutations in the target protein [18]. This, however, is not the only option for the cancer cells to elude the drugs. Compensatory changes in the signaling pathways of treated cancer cells can bypass the drug mediated inhibition. They can restore the signaling pathway activity inhibited by the targeted drug or activate signaling components which elicit the same phenotypic consequences as the original pathway.

Signaling redundancies and interconnections through pathway crosstalk have been identified as contributors to drug resistance [12]. Negative feedback loops, cross inhibition, cross activation and pathway convergence are hallmarks of molecular mechanisms which connect signaling pathways through activated components. These mechanisms govern the integration of simultaneous signaling events, limit their extent and duration [19] and balance outcomes to assure cellular stability and homeostasis. They are crucial for the maintenance of normal cell functions and for the dynamic and adaptive responses to extracellular signals [20, 21]. These balanced situations can be disturbed when a particular signaling component is inhibited by drug treatment. Not only an oncogenic kinase activity can be affected, but also regulatory loops can become disrupted. Negative feedback loops might be subjected to a relief of repression and the up-regulation of pathway components or the activation of compensatory pathways might result. This can cause the activation of other e.g. protein kinases which phosphorylate the same substrates as the initially inhibited kinase and thus compensate for its activity and the activity of downstream effectors. Even if a crucial oncogenic kinase is suppressed by the inhibitor, the tumor cells can exploit the interconnections between signaling pathways, switch their signaling network and evolve drug resistance derivatives [22].

A number of examples for molecular mechanisms which mediate signaling redundancy, pathway crosstalk and feedback inhibition have been studied in detail [20]. They explain aspects of compensatory signaling and the emergence of inhibitor resistance. Negative feedback loops in oncogenic kinase cascades can play central roles in drug resistance. When an inappropriately activated kinase is inhibited, the immediate response of the cell might be growth arrest or even the induction of apoptosis. However, if the kinase inhibition results in the relief of negative feedback inhibition, this might cause the activation of functionally related kinases and pathways. The phenotypic consequence would be a transient inhibition of growth and the resumption upon the activation of the compensatory loop.

Receptor tyrosine kinases (RTK) are initiators of intracellular signaling and their deregulation contributes to cancer formation. The EGF receptor family e.g. signals through ligand induced receptor dimerization. Activating mutations in the EGFR are found in 25% of non-small cell lung carcinomas (NSCLC), and ErbB2 amplifications in 20% of metastatic breast cancer. ATP-competitive TKIs (erlotinib, gefitnib, and lapatinib) are in clinical use to inhibit ErbB signaling in these cancers. Acquired resistance to TKI directed against the EGFR in these tumors is partially due to gatekeeper mutations [23], but pathway redundancy has emerged as an important alternative mechanism. The inhibition of the EGFR in NSCLC e.g. can be compensated by the enhanced expression of the MET receptor. This receptor can activate similar effector cascades as the EGFR, but is not affected by the TKI directed against the EGFR [24]. Other RTK and activation mechanisms can be involved. Compensatory resistance can be caused by the activation of IGF-1R through the loss of a negative regulator, IGFBP3. This efficiently promotes PI3K signaling and can compensate for EGFR inhibition in squamous carcinoma cells [21].

The Ras-ERK (extracellular signal-regulated kinase) and PI3K (phosphatidylinositol 3-kinase)- mTOR (mammalian target of rapamycin) signaling pathways provide the major mechanisms for the regulation of cell survival, differentiation, proliferation, metabolism and motility in response to extracellular ligands. The signaling events of the Ras-ERK and PI3K-mTOR pathways intersect, regulate each other and their downstream functions. The extent of this crosstalk is clinically important and the inhibition of the PI3K-mTOR kinase provided insights into the principles of compensatory signaling.

A mTOR-dependent negative feedback loop normally results in the inhibition of PI3K signaling. When mTOR is inhibited, the disruption of this negative feedback loop enhances the activity of PI3K and its effector AKT. This counteracts the antiproliferative effects of mTOR inhibition [25]. In glioblastoma patients, this is reflected by the modest response towards mTOR inhibition in the presence of PTEN inactivating mutations and an enhanced AKT activity.

AKT inhibition also affects the FOXO transcription factors. They are substrates of the AKT kinase and their phosphorylation by AKT promotes 14-3-3 binding and their cytosolic sequestration. AKT inhibition therefore promotes the nuclear localization of FOXO and the transcription of FOXO target genes, e.g. ErbB3, IR, and IGF-1R. In breast cancer cells, this results in increased ErbB2/ErbB3 signaling and the compensatory activation of the ERK cascade [26, 27]. Combined inhibition of mTOR kinase and the induced RTKs therefore seems a promising approach abolish AKT signaling and prevent resistance formation [28].

The PI3K/mTOR pathway is functionally connected with Jak/Stat signaling. This observation was made when breast cancer cells were exposed to inhibitors of PI3K. In these cells, the inhibition of PI3K coincided with the activation of Jak2. Jak2 in turn phosphorylated and activated Stat5, and Stat5 transactivated its target gene IL-8. The secretion and autocrine activation of the IL-8 receptor compensated for the inhibition of PI3K. An effective therapy has to take this into consideration and would ideally preclude the compensatory mechanism. A combination therapy of PI3K and Jak2 inhibition would seem appropriate [29].

Negative feedback regulation has also been observed in the extracellular signal–regulated kinase (ERK) cascade.

This signaling pathway is initiated by RAS activation, proceeds through the effectors A-RAF, B-RAF and C-RAF, the activation of the ERK pathway and regulates the transcription of genes governing cell cycle progression [20]. Inhibition of B-RAF in cells with active RAS caused the unexpected activation of the ERK cascade by favoring the formation of B-RAF C-RAF heterodimers [30]. B-RAF selective inhibitors might thus be detrimental for patients with activating RAS mutations.

4. THE JAK/STAT PATHWAY AND THE PRESENCE OF PERSISTENTLY ACTIVATED STAT3 IN CANCER

The Jak/Stat signaling pathway constitutes one of the mechanisms which relay extracellular signals from the cell surface to the nucleus. Small peptide ligands, present in the extracellular milieu, are being recognized by specific trans-membrane receptors. This ligand receptor interaction triggers the intracellular activation of signaling molecules and finally results in changes in the patterns of gene transcription.

The Jak-Stat pathway regulates many essential cellular functions, e.g. cell growth, differentiation and metabolism, and its activation can be observed in diverse cell types and organs [31]. The Stat protein family consists of seven members (Stat1, 2, 3, 4, 5a, 5b and 6). These proteins are related and characterized by a common configuration of structural and functional domains. They range in lengths from 750 to 850 amino acids. The functional domain present at the amino terminus is a tetramerization domain. Furthermore, there is a coiled-coil and a central DNA-binding domain (DBD). This is followed by a linker domain, the SH2-domain required for dimerization and the carboxyl terminal transactivation domain, an interaction domain for co-activators and co-repressors. The TAD is responsible for the tissue and cell type specificity of Stats and subject to additional secondary modifications [32].

Gene deletion in mice have shown that Stat3 and Stat5 are required for embryonal development and that their inactivation is lethal early on. Stat1, Stat2, Stat4, and Stat6 have functions in more specialized cell types and play major roles in immune regulation [33]. The phosphorylation of Stats on a particular tyrosine residue located in the carboxyl terminal region is prerequisite for the transition from the latent to the active state. This phosphorylation step can be accomplished by various tyrosine kinases, including the receptor associated Janus family kinases (Jak), the EGFR or other members of the growth factor receptor kinases, or by cytoplasmic tyrosine kinases, e.g. members of the Src family. The phosphorylation of the Stat proteins allows them to form dimers, these dimers result from the reciprocal interaction of the SH2 domain with the tyrosine phosphorylated domain of the dimerization partner [34]. It is also accompanied by a conformational change which exposes a nuclear localization signal (NLS). This NLS is located within the DBD of the Stat dimers [35]. Its exposure allows the interaction with importin-a and the translocation of the dimer into the nucleus. In the nucleus Stats bind to GAS-motifs: gamma interferon activated consensus sequences of the composition TTCNNNGAA. They are part of target gene promoters.

In addition to the canonical transactivation function of Stats, non-canonical functions have been discovered. Both, phosphorylated dimers and non-phosphorylated monomers of Stat3 and Stat5 can assume functions in the cytoplasm, the mitochondria and in the nucleus. They can regulate the activities of mitochondria, can maintain the integrity of the Golgi apparatus and the rough endoplasmatic reticulum, they can modify histones and change the state of the heterochromatin and they can influence the dynamics of the cytoskeleton [31, 36, 37]. Jak-Stat signaling is a widely used mechanism and has been found in many organs. Among them are the mammary gland, lymphocytes, adipocytes, neuronal cells, cardiomyocytes, hepatocytes, eye cells, stem cells. Deregulation of Stat signaling is most disruptive and contributes to many disease states including the emergence and progression of cancer cells [31, 38, 39].

Stat3 and Stat5 have been described as potent oncogenes [40-43] and as a promising target for cancer therapy [44]. Upon ligand mediated activation of the Jak/Stat pathway the phosphorylation and transactivation activity of Stat3 is transient and the de-activation is tightly regulated. In most tumor cells the activation persists and aberrantly activated Stat3 becomes oncogenic [19].

5. COMPENSATORY KINASE SIGNALING AND THE ACTIVATION OF STAT3

The role of Jak/Stat signaling in tumorigenesis have defined Stat3 as a promising drug target [41]. Stat3 is an unconventional drug target without binding pockets for small molecular weight ligands. This has led to attempts to discover and develop new classes of drugs [44-46] and to attempts to block upstream activating kinases [47, 48]. In the course of these experiments a number of interesting observations were made [49].

Stat3 and Stat5 are activated in head and neck squamous cell carcinoma (HNSCC). Since Stat activation occurs as a downstream event of a number of signal transduction pathways, e.g. the epidermal growth factor receptor (EGFR), alpha7 nicotinic receptor, interleukin-6 receptor and erythropoietin receptor, it is not immediately obvious which tyrosine kinase is responsible for Stat3 activation and should be inhibited [49]. Jak2 and c-Src are reasonable candidates. Src kinase inhibition with the drug dasatinib, resulted in reduced Stat3 and Stat5 activation and reduced cell proliferation. The inhibition of c-Src caused a persistent downregulation of P-Stat5, but only a transient inhibition of P-Stat3. The activation of Stat3 resumed within several hours [50].

The restoration of Stat3 activity in the presence of the c-Src inhibitors is dependent upon another kinase. Compensatory induction of Jak kinase activity caused Stat3 activation and the survival and proliferation of the cancer cells [51, 52]. The feedback loop, which causes the activation of Stat3 in the continuing presence of the c-Src inhibitor, makes use of a most interesting molecular mechanism. It was found that c-Src inhibition results not only in the downregulation of Stat3, but simultaneously in the in the downregulation of activated Stat5. Activated Stat5 induces its target gene SOCS2 (suppressor of cytokine signaling 2) which is a negative regulator of Jak2. The negative regulation of Jak2 by SOCS2 subsequently results in Stat3 de-activation. If Stat5 is de-activated by the c-Src inhibitor and SOCS2 expression is reduced, Stat3 can be reactivated. This is most likely

accomplished by Jak2 activity. These results suggest that the combination of Jak and c-Src inhibitors might counteract the feedback loop which causes Stat3 reactivation and should be used in therapeutic settings [1, 21, 52].

Compensatory Stat3 activation upon the inhibition of an upstream kinase does not seem to be limited to HNSCC and the action of c-Src. We have investigated the consequences of Bmx inhibition in glioma cells and have made similar observations (von Manstein und Groner, unpublished results). Glioblastomas are aggressive tumors which are difficult to treat due to the presence of tumor propagating glioblastoma stem cells (GSCs). Stat3 activation in these cells has been identified as a prerequisite for GSC maintenance. Activation of Stat3 in GSC is affected by the non-receptor tyrosine kinase BMX. It phosphorylates Stat3 and thus enables GSC self-renewal and tumorigenicity [53].

Bone marrow tyrosine kinase gene on chromosome X (Bmx or Etk), belongs to the Tec family of non-receptor tyrosine kinases, a gene family with five members (Btk, Itk/Emt/Tsk, Rlk/Txk, Tec, and Bmx/Etk) [54]. The Tec family kinases are similar to the Src family of kinases and contain a carboxy-terminal kinase domain, and SH2 and SH3 domains; in addition, they comprise a Tec homology domain and a pleckstrin-homology (PH) domain. Tec family kinases associate with the plasma membrane, recruited by lipid molecules or protein motifs, e.g. phosphatidylinositol (3,4,5)-triphosphate (PIP3), the Focal Adhesion Kinase (FAK), caveolin-1, heterotrimeric G-protein subunits, Protein Kinase C (PKC) or F-actin. Membrane bound Tec kinases are activated by cell surface receptor-associated signaling complexes, e.g. receptor tyrosine kinases (RTK), cytokine receptor-associated kinases, integrin associated kinases, G-protein coupled receptors (GPCR) and Src family kinases (SFK) and involved in the regulation of gene expression, calcium mobilization, actin cytoskeleton organization and cell survival [54]. Bmx is expressed in granulocytes, monocytes, cells of epithelial and endothelial lineages, as well as brain, prostate, lung and heart. Upstream regulators of Bmx are c-Src, FAK and PI3K. Downstream targets of Bmx include Akt, Stat3 and p21 activated kinase 1 (PAK1) [55].

Bmx can regulate processes like adhesion, motility, angiogenesis, proliferation and differentiation and a role in tumorigenicity has been assigned. It is overexpressed in metastatic breast [56] and prostate cancers [57]. Bmx may be a component of the adaptive compensatory mechanism activated by androgen ablation in prostate cancer patients causing their resistance to anti-hormone therapy [57]. For these reasons Bmx is a promising target structure for cancer drug development.

We detected the expression of Bmx in the human breast cancer cell lines MDA-MB-231 and MDA-MB-468. Bmx activation can be shown by phosphorylation of the tyrosine residue 566 (Y566) and was found in the murine glioma cell lines Tu-2449 and Tu-9648. Homogeneous expression and activity of Bmx was observed in the entire population of the cultured murine glioma cells. We studied the hierarchy and the interconnections of enzymes leading to the activation of Stat3 in these tumor cell lines and downregulated the expression of tyrosine kinases by RNAi. We also treated the cells with specific kinase inhibitors. Addition of canertinib, an ATP-competitive Bmx inhibitor, to glioma cells for six hours, resulted in the strong decline of P-Stat3, indicating that Stat3 is a substrate of this kinase in these cells. However, 48 hours after canertinib addition, Stat3 phosphorylation at tyrosine residue 705 is being restored (Fig. **1**). The recovery of the cells and the restoration of Stat3 phosphorylation, however, are accompanied by cell death (Fig. **2**). We suggest that the inhibition of Bmx triggers a compensatory Stat3 activation mechanisms in a subfraction of the cells, possibly through the relief of negative feedback inhibition. An accessory tyrosine kinase might become activated and replace the Stat3 activating function of Bmx (Fig. **3**). This adaptive resistance could possibly also involve endocrine remodeling and the secretion of autocrine factors [22]. We are further investigating the underlying mechanisms through the combination of tyrosine kinase inhibitors and the sequestration of soluble factors by antibodies.

6. POSSIBLE ESCAPE ROUTES: MECHANISMS OF STAT3 ACTIVATION BASED UPON INTRA-CELLULAR CIRCUIT REWIRING AND ON INTER-CELLULAR COMMUNICATION IN THE TUMOR MICROENVIRONMENT

The end point of compensatory signaling in the examples discussed above is the maintenance of Stat3 phosphorylation. The persistent activation of Stat3 is required for tumor cells of many different origins to survive and to grow [58].

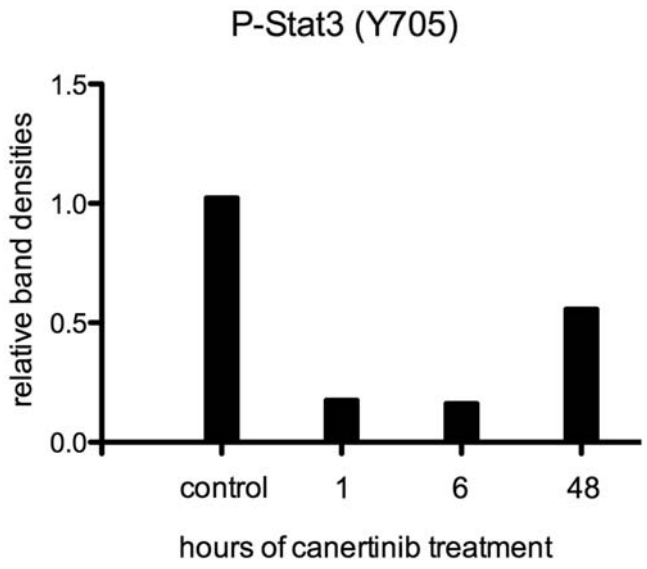

Fig. (1). The inhibition of the Bmx kinase with canertinib in Tu-2449 glioma cells causes a transient loss of Stat3 activation. Tu-2449 murine glioma cells were treated with 10 μM of the Bmx kinase inhibitor canertinib for 0 to 72 hours or with DMSO for 72h as a control. At the times indicated protein extracts were obtained from the treated cells and activated P-Stat3 was visualized and quantitated upon Western blotting. Shown are the relative band densities normalized to the loading control beta-actin. After 1 hour of treatment, deactivation of Stat3 was observed. Stat3 phosphorylation resumed after 48 hours in the continued presence of the Bmx inhibitor.

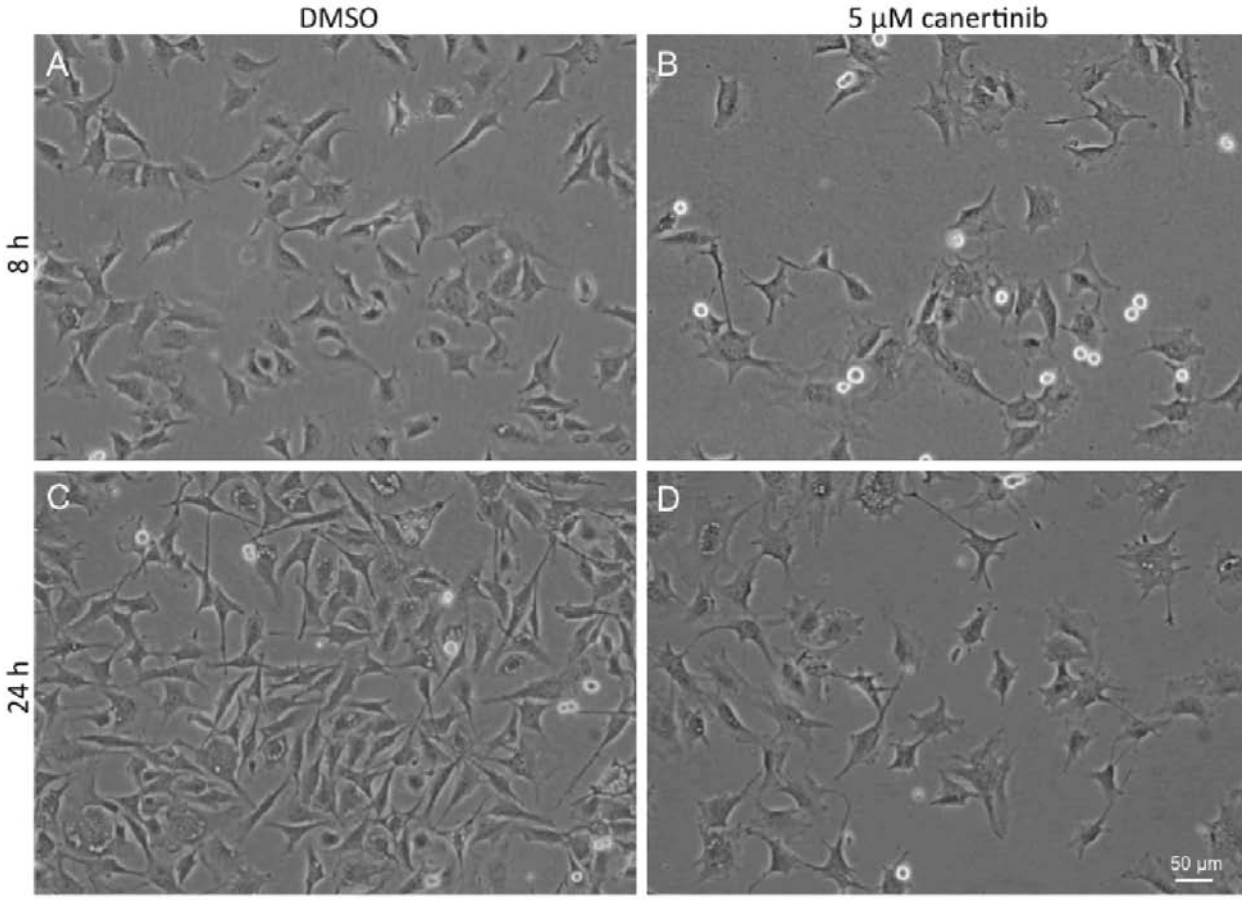

Fig. (2). The inhibition of the Bmx kinase with canertinib in Tu-2449 glioma cells initially causes cytopathic and cytotoxic effects, but the cells seem to recover after extended treatment periods. Tu-2449 cells were treated with 5 µM canertinib for 8 and 24 hours, photographed and compared to DMSO-treated control cells. **A**, **C** cells treated with DMSO for 8, 24 hours; **B**, **D** cells treated with 5 µM canertinib for 8, 24 hours.

Multiple molecular mechanisms have been found which influence Stat3 activation and de-activation and thus contribute to the regulation of the extent and the duration of Stat3 signaling. Mutations which affect these regulatory components, and which result in changes in quantitative aspects of Jak-Stat signaling, are initiators of tumorigenesis. The mutations can enhance the functions of components which are involved in the Stat3 activation steps, e.g. tyrosine kinases, or diminish the functions of components which are involved in the downregulation of Stat3 signaling, e.g. protein tyrosine phosphatases. Gain of function mutations can also affect cytokine and growth factor receptors, resulting in their ligand independent activation. Mutations have also been found in the Stat3 molecule itself, which render the molecule hyperactive through enhanced and prolonged tyrosine phosphorylation. The autocrine or paracrine secretion of cytokines, most notably IL-6 [41], is a widespread property of tumor cells. Reduced downregulation of phosphorylated Stat3 can caused by the to the diminished expression of tyrosine phosphatases, mutations which inactivate these enzymes, or epigenetic silencing of e.g. SOCS molecule encoding genes. In addition, the post-transcriptional inhibition of PIAS3 expression has been observed. Deletion mutations can affect the lymphocyte adaptor protein, LNK. Stat3 variants which exert an endogenous transactivation ability have been detected in T-cell large granular lymphocytic leukemia cells, myeloid malignancies [59] and human inflammatory hepatocellular adenomas [19, 60].

The type of mutations found in Jak-Stat pathway components, i.e. gain or loss of function mutations, often allow to draw conclusions about the molecular mechanisms underlying the Stat deregulation. In addition to mechanisms with a mode of action endogenous to the tumor cells, mechanisms have been identified which are based on signal exchanges between tumor cells and their surrounding normal cells. The cells within the tumor microenvironment interact through the secretion of factors and establish a network of reciprocal communication. Crosstalk and feedback loops have been identified. Important molecules in the communication between tumor cells and normal cells in the tissue microenvironment are sphingosine 1-phosphate and the sphingosine 1-phosphate receptor. They are coupled to the activation of NF-kB and Stat3 through the induction and paracrine action of IL-6. This signaling cascade can cause the persistent activation of Stat3 and trigger cellular transformation [61-63].

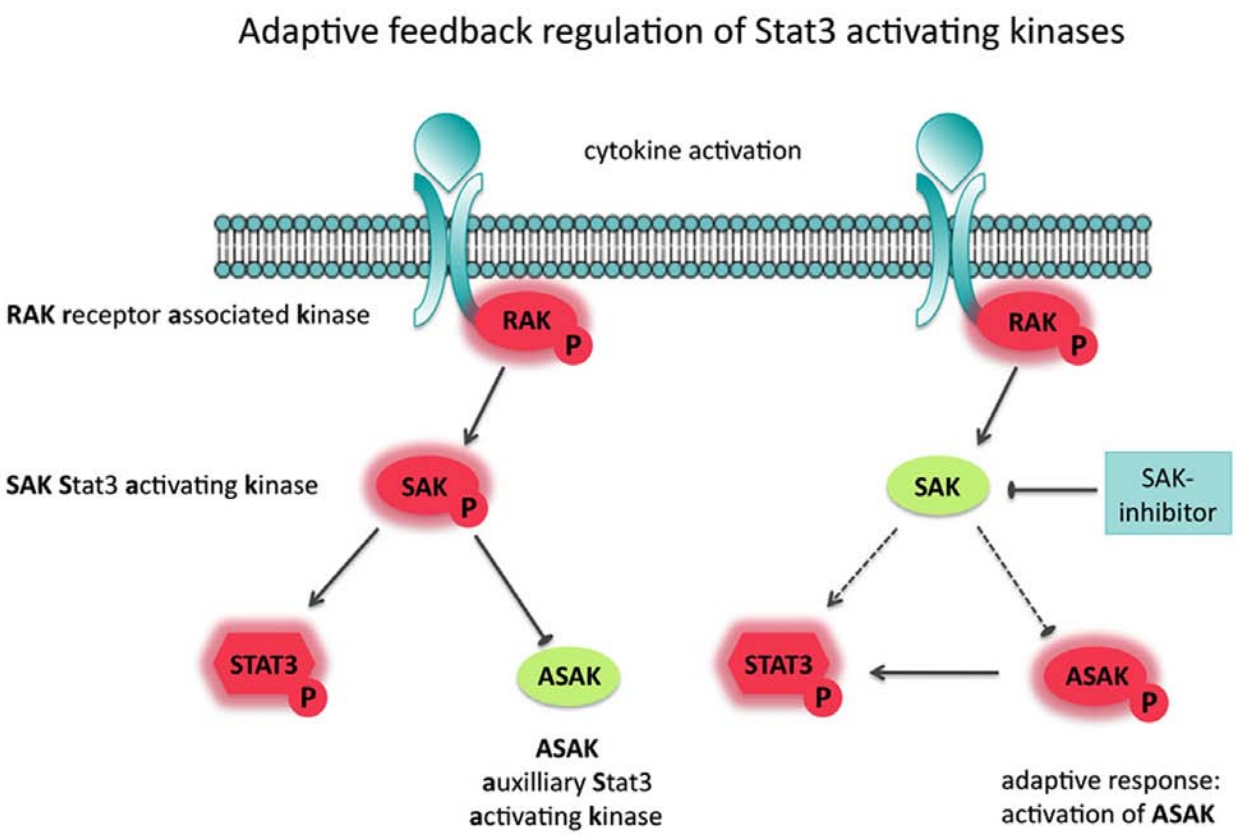

Fig. (3). Model for the adaptive resistance of tumor cells towards targeted kinase inhibitors: mediation through the relief of negative feedback regulation. The inappropriate activation of cell surface receptor induced signaling pathways can cause the oncogenic transformation of cells. In normal cells the activation of signaling pathways is transient and multiple negatively acting components limit the extent and the duration of external ligand induced signals. However, negative feedback regulation not only plays a part in the limitation of signal strength, but it is also an important mechanism which allows for compensation of the loss of signals and protects cells from the absolute dependency from individual signaling molecules. In the model shown here, two kinases are able to activate Stat3 through tyrosine phosphorylation. The Stat3 activating kinase (SAK) is normally active upon receptor ligand engagement and causes Stat3 phosphorylation. SAK has a dual function and suppresses the activity of an auxiliary Stat3 activating kinase (ASAK), also possibly through its phosphorylation. Specific inhibition of SAK, by the SAK inhibitor, causes the temporary loss of Stat3 activation. The simultaneous relief of repression of ASAK causes an adaptive response, leads to the resumption of Stat3 activation and the persistence of the Stat3 dependent transformed cells. The loss of negative feedback can thus promote oncogenic signaling and cancer cell survival. A combination of inhibitors, targeting SAK and ASAK, could become beneficial in this situation.

The sphingosine-1-phosphate receptor 1 (S1PR1) is a G-protein-coupled receptor and is recognized by its ligand, the lysophospholipid S1P. The receptor is strongly expressed in tumor cells with activated P-Stat3 [64]. The S1PR1 gene, in turn, is a target gene for P-Stat3. A positive feedback loop is established through the enhanced S1PR1 expression by P-Stat3 and the activation of Stat3 by this receptor and Jak2 [62].

NF-kB is another transcription factor often hyper-activated in tumor cells. NF-kB induces anti-apoptotic target genes. Very much like Stat3, NF-kB is only transiently induced in normal cells. It responds to cell surface receptors which are activated by pro-inflammatory stimuli, e.g. IL-1b and TNF. The persistent activity of NF-kB in tumor cells can be mechanistically linked to Stat3. Stat3 enhances the nuclear retention of NF-kB. It does so through RelA acetylation. This enzymatic reaction is catalyzed by the Stat3 associated acetyltransferase p300 and results in the inhibition of the nuclear export of NF-kB [63]. The prolonged NF-kB transactivation activity, caused by P-Stat3, results in the upregulation of the IL-6 gene. IL-6 is secreted from the cells, activates the IL-6 receptor and thus enhances the levels of P-Stat3.

The long lasting activation of Stat3 as a result the S1PR1-Stat3-NF-kB-IL-6 cascade affects cells which are hallmarks of chronic inflammation and which establish precursor states for cancer initiation, development and progression [65]. Targeted drugs which interrupt this chain of events are most promising therapeutic agents and could possibly prevent tumor formation and subsequent tumor progression [66].

The cytokine IL-6 plays a major role in the process of tumor initiation and progression to the metastatic state. High levels of IL-6 have been detected at the invasive front of primary human breast cancers. The secretion of IL-6 and the process of tumor invasion are tightly connected. It is thought that the contribution of activated Stat3 to the process of

tumor progression might involve the recruitment of myeloid cell, the induction of angiogenesis and the finally the extravasation of metastatic cells. Several steps can be possibly blocked and the inhibition of the IL-6 receptor, of Jak1/2 kinases or of the Stat3 molecule directly, have been taken into consideration. These inhibitory actions could become therapeutically beneficial and might exert effects on tumor supportive stroma, e.g. angiogenesis, fibroblast infiltration and prevent myeloid suppressor cell recruitment [67]. Gliomblastoma cells have been used as a model to study the inhibition of IL-6 and its receptor. RNA interference experiments showed that the downregulation of IL-6 or the IL-6 receptor suppressed the growth and neurosphere formation capacity, and at the same time increased their apoptosis. These results were confirmed with inhibitors targeting Stat3 [38, 68]. Additional drugs and drug candidates which target components of the IL-6/Jak-Stat3 axis are being investigated and developed [41]. Inhibition of Stat3 also counteracts the immunosuppressive tendencies in the tumor micro-environment and and thus could possibly improve the results of conventional cancer therapy. IL-6 and IL-6 receptor can be inhibited by specific antibodies, and small molecular weight compounds can act as Jak2 and Stat3 inhibitors.

Myeloid cells have the capacity to promote metastases at distant organs. This process was also mediated by S1PR1-Stat3 upregulation. S1PR1 activation by S1P caused the induction of Stat3 in tumor cells. These cells then secrete factors that are able to activate the S1PR1-Stat3 pathway in the cells at a premetastatic site. This allows the formation of premetastatic niches [69].

Sphingosine kinase 1, SphK1, is the enzyme that catalyzes the intracellular formation of sphingosine 1-phosphate, the ligand for the S1PR1. A specific SK1 inhibitor appears well suited to disrupt the S1PR1-Stat3-NF-kB-IL-6 axis. This was investigated in an experimental system of progression from chronic inflammation to colon cancer. Sphingosine kinase 1 and S1-P are being expressed in cases of chronic intestinal inflammation and colitis-associated cancer (CAC). The prodrug FTY720 inhibits SphK1 and reduces S1PR1 expression. This results in the disruption of the S1PR1-Stat3-NF-kB/IL-6 amplification cascade and impeded the development of CAC [65].

7. DISCUSSION

Drug resistance, inherent and acquired, has been a serious problem in cancer therapy [70]. It limits the therapeutic effects of conventional chemotherapy, but it also restricts the benefits of targeted agents. Cancer cells are most adaptive and respond to drug treatment with adjustments of their signaling networks. Functional redundancies, feedback regulation and cross-talk are mechanisms which are mobilized by cancer cells to ensure their survival in the continuing presence of drugs [21, 22]. Additional insights into the mechanisms of drug resistance are being gained. Resistance mechanisms initially recognized, based on drug transport and metabolism, are being complemented by mechanisms based on mutations in target proteins and adaptations in signaling pathways. These mechanisms are not mutually exclusive and could possibly act in parallel.

We now know that cellular functions are regulated by a network of effector pathways. These pathways interact with each other and maintain cellular stability, but also trigger proliferation, differentiation, migration and survival. These observations have to be taken into consideration when targeted therapeutics are being deployed in the clinic. Selective inhibition of critical signaling components in tumorigenic pathways have had remarkable benefits for patients and the task now is to extend the duration of these benefits. The mechanisms how cancer cells transiently and persistently respond to targeted therapeutics, how they avoid the toxic effects of drugs and prevent curative results, will become a major area of cancer biology. The acquired resistance of cancer cells has to be approached from a signaling network perspective and take into account the insights into the regulatory mechanisms which establish interconnections and adaptations between effector pathways.

This task can exploit the detailed knowledge of signaling which has accumulated over the past years. Genetic and biochemical studies and their integration into systems biology have yielded most valuable information on which rational drug therapies can be based in the future [11]. It will become necessary to initially identify oncogenic drivers and initiate treatment with a drug targeting this molecule. Once resistance occurs, the determinants of resistance, either cell autonomous pathway switches or intercellular communication routes, will have to be investigated and characterized. The use of siRNA screens and the systematic evaluation of available pharmacological inhibitors of kinase activities, will become helpful. We will also have to learn about the potential of cells to adapt to drug pressure. A large fraction of tumor cells dies upon exposure to drugs targeting activated oncoproteins and only a small fraction persists. Stem cells are predestined to change their genetic programs and phenotypes in response to environmental clues, a property which might be related to drug adaptation.

In the clinic, profiling of DNA mutations and pathway activities in the tumor cells of each individual patient will be a critical determinant for the choice of drug for first line treatment. Subsequently, upon the development of resistance to this drug, the appropriate combinatorial treatment will again rest on a molecular diagnosis. The limited number of essential pathways [2], and their well defined components, might indicate that the search for resistance determinants might not be prohibitively complex. If it is possible to assign particular escape routes to the inhibition of individual oncoprotein drivers, the combined inhibition of driver components and of components commonly activated in the emergence of drug resistance will be most promising. Monitoring of tumors at the signaling level during treatment will be required to achieve a durable response through the combination of signaling blocks that can not be bypassed. Although this strategy seems tedious and expensive, it probably will eventually lessen the economic burden of cancer [71].

The principle of compensatory Stat pathway activation also defines new priorities in drug development. Since Stat3 is the endpoint of the signaling cascade in the original tumor cells and in the drug adapted, resistant cells, Stat3 should be a most favorable drug target. This emphasizes the need to develop suitable drugs for such unconventional drug targets as Stat3 [72]. Direct interference with the functions of Stat3,

Stat5 and NF-k or other oncogenic transcription factors should be pursued with great vigor [46, 73].

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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