Personalized Cancer Medicine: From Molecular Diagnostics to Targeted Therapy with Natural Products

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- chemotherapy
- comparative genomic hybridization
- drug resistance
- pharmacogenomics
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Abstract

Personalized cancer medicine aims to develop individualized treatment options adapted to factors relevant for the prognosis of each patient. Molecular biomarkers are required to predict the likelihood of an individual tumor's responsiveness or of toxicity in normal organs and to advise optimized treatments with improved efficacy at reduced side effects for each cancer patient. In the present review, we present a concept, which takes advantage of methods of molecular diagnostics to identify predictive markers at the DNA, mRNA, and protein levels. Markers with prognostic value concerning treatment response and patient survival can then be used as targets to develop optimized drugs. We focus on three examples to illustrate this strategy: (i) chemoselective treatment of tumors with 9p21 deletion by L-alanosine, (ii) treatment of multidrug-resistant P-glycoprotein-expressing tumor cells by non-cross-resistant natural products or by inhibitors of P-glycoprotein to overcome multidrug resistance, and (iii) natural products that inhibit the epidermal growth factor receptor (EGFR) in EGFR-overexpressing tumor cells.

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Molecular Diagnostics of Prognostic Markers

Resistance to chemotherapy and the concept of individualized treatment

The basis of multicomponent cancer therapy is the view that cancer cells resistant to one drug remain susceptible to other drugs. Clinically, the success of combination treatments is frequently limited due to the development of broad-spectrum or multidrug resistance (MDR). Since most established cytostatic drugs lack sufficient tumor specificity, normal tissues are also affected leading to severe side effects. This prevents the application of sufficiently high doses to kill resistant tumor cells or resistant tumor stem cells. Thereby, drug resistance develops causing treatment failure. Novel strategies to broaden the narrow therapeutic range by separating the effective dose and toxic dose would be of great benefit for the patients.

Whereas the statistical probability of therapeutic success is well-known for larger groups of patients from clinical therapy trials, it is, however, not possible to predict how an individual tumor will respond to chemotherapy. The question arises as to which particular cytostatic agent and which combination of substances is most suited for an individual tumor. Although clinicopathological prognostic factors such as tumor size, lymph node, and far distance metastases are valuable for the determination of the prognosis of larger cohorts, they are less helpful for individualized cancer treatment. Further biomarkers are required to predict the likelihood of an individual tumor's responsiveness or of toxicity in normal organs of each patient.

Clinically, it has been known for many years that the same doses of a medication cause considerable heterogeneity in efficacy and toxicity across human populations [1,2]. This heterogeneity can lead to unpredictable life-threatening or even lethal adverse effects in patients who react hypersensitively [3,4]. The interindividual variability in drug response cannot satisfactorily be explained by clinical factors such as renal and liver function, patient's age and comorbidity, lifestyle, or co-medication and compliance of the patient. Therefore, molecular factors come into the center of interest.

In the past decades, enormous efforts have been undertaken to predict drug resistance *in vitro* [5, 6]. The idea was to determine sensitivity or resistance beforehand to be subsequently able to

choose the clinically most effective treatment for each individual patient. The methods available at that time, however, were not established for clinical routine diagnostics. In the 1990s, attempts were made to test the drug response of cancers by determination of the expression of resistance proteins. However, it was not possible to define consensus recommendations for the standardized detection of resistance proteins expressed in low amounts in tumors with low degrees of drug resistance [7–9]. Hence, this approach was also not realized in clinical routine. Another important reason is that no single mechanism can sufficiently explain resistance to therapy [10]. While the concept of individualized therapy itself traces back to the 1950s [11], the advances in cell and molecular biology have only recently opened new avenues for the characterization of drug-resistant tumors. Still, the transfer of such techniques from the bench to the bed is an unfulfilled requirement. Nevertheless, current progress in molecular biology gives reason to believe that molecular approaches will significantly improve individual tumor therapy.

In the present overview, we focus mainly on our own efforts in this thriving field of research. We searched for novel markers to predict the responsiveness of tumors to chemotherapy and the survival chances of patients at the level of DNA, mRNA, and proteins (**Fig. 1**). Promising candidate markers from these analyses were then taken to identify novel compounds derived from natural origin, which specifically target these markers. In a recent survey from the National Cancer Institute (NCI, USA), it was convincingly shown that the majority of cancer drugs brought on the market during the past half century is derived from natural sources or is based on principles of action taken from nature [12, 13]. Therefore, we have concentrated on natural products [14–16]. For general overviews in the field of personalized cancer medicine, the reader is also referred to comprehensive reviews in the literature [17–22].

A special feature of our concept is the combination of methods of molecular diagnostics to identify prognostic markers with natural product research to identify inhibitors for these markers. Normally, both areas of research are separated from each other. An integration of both areas in one interdisciplinary approach may open new avenues for the development of novel treatment options for cancer.

Predictive DNA markers

Classical cytogenetics: Clear cell renal cell carcinoma is a tumor type with a poor prognosis and low responsiveness towards chemotherapy. Less than 50% of patients can be cured by other therapies such as surgery. To evaluate the prognostic significance of cytogenetic findings in clear cell renal cell carcinoma, the results of classical cytogenetic staining techniques (DAPI-staining and G-banding) and the results of 118 primary RCCs were evaluated in relation to classical indicators of prognosis and overall survival [23]. Losses at the short arm of chromosome 3 (3 p) were most prevalent and included 32 monosomies of chromosomes 3 and 84, structural aberrations involving unbalanced translocations resulting in duplication of sequences at the long arm of chromosome 5 (5 q). Patients with the gain of band 31 to the end of chromosome 5 (5q31-qter) had a significantly better outcome than those without this aberration ($p = 0.001$). There was no association between the gain of 5 q and any of the well-known variables for prognosis, including low versus high clinical stage and grade of malignancy. Among additional chromosomal aberrations, loss of chromosome 9/9 p was associated with distant metastasis at diagnosis ($p = 0.006$). The data indicate that the gain of 5 q identifies a clinically favorable cytogenetic variant of clear cell renal cell carcinoma and demonstrate the impact of specific chromosome aberrations as additional prognostic indicators in clear cell RCC. The usefulness of cytogenetics for prognosis of a wide variety of hematopoietic and solid cancers has been demonstrated during the past two decades [24–33].

Comparative genomic hybridization (CGH): CGH is an advanced cytogenetic method to analyze unbalanced genomic aberrations, i.e., gains and losses of genetic material (amplifications and deletions). Balanced aberrations (translocations and inversions) cannot be visualized by CGH. In a pilot project, we analyzed whether it is possible to dissect drug-resistant tumor cells from sensitive ones by comparative genomic hybridization. Ten T-cell acute lymphoblastic (T-ALL) CEM cell lines selected for resistance towards methotrexate, doxorubicin, vincristine, or hydroxyurea, respectively, and parental drug-sensitive CCRF-CEM cells were analyzed [34]. Most genomic imbalances were not specific for drug resistance, as they were found in both parental and drug-resistant lines. We were concerned with those imbalances, which were specifically present in drug-resistant, but not in drug-sensitive cells. All methotrexate-resistant cell lines were characterized by an enhancement or an amplification of 5q13. The methotrexate resistance-conferring dihydrofolate reductase (*DHFR*) gene is located at this locus. Gain of *DHFR* was verified by PCR analyses. Some but not all methotrexate-resistant cell lines showed enh(14q21qter) and amp(5p13p15.2). These two loci harbor the methylenetetrahydrofolate dehydrogenase (*MTHFD1*) and 5′-methyltetrahdrofolate-homocysteine methyltransferase reductase (*MTRR*) genes, both of which are involved in folate metabolism. Their gain indicates a role in methotrexate resistance. A loss of 4q35 was found in two methotrexate-resistant sublines and in the doxorubicin-resistant cells, where the proapoptotic caspase-3 gene is located. The thioredoxin (*TXN*) locus 9q31 was also enhanced in the doxorubicin-resistant cell line. Furthermore, 2p22pter was increased in hydroxyurea-resistant CEM cells. Ribonucleotide reductase polypeptide M2 (*RRM2*), which confers resistance to hydroxyurea, resides at this locus.

Furthermore, genomic imbalances were investigated in 15 T-cell acute lymphoblastic leukemia cell lines using CGH [35]. In addition, the *in vitro* response to the cytostatic drug doxorubicin was evaluated by means of a growth inhibition assay. The most frequent genomic imbalance (gain of 6q23) was shared by 9 of the 15 cell lines. This chromosomal locus harbors the *C-MYB* oncogene. A significant loss of 18q23 was observed in eight lines. Seven of the cell lines were characterized by a loss of the entire short arm of chromosome 9 or parts of it with 9p21 as a minimal band of overlap. This locus contains the tumor suppressor genes *INK4A, INK4B, ARF* and the *MTAP* gene. Interestingly, cell lines with a 9p21 deletion exhibited twice the number of gains and 1.6 times the number of losses per line as compared with the cell lines without this deletion. Based on the dose-response curves of the cell lines for doxorubicin, eight doxorubicin-sensitive cell lines had an inhibition concentration 50% (IC_{50}) < 10 nM (CCRF-CEM2, JURKAT, KE-37, MOLT-3, MOLT-4, P12-Ichikawa, PEER, and RPMI-8402) and seven doxorubicin-resistant cell lines had an IC_{50} > 10 nM (BE-13, CCRF-CEM1, HUT-78, J-Jhan, Karpas-45, MOLT-17, and PF-382). The average number of copy number alterations (CNAs) per cell line was higher in the sensitive than in the resistant group.

Eight cell lines newly established from glioblastoma multiforme were also examined by CGH for their patterns of genomic imbalance [36]. The total number of CNAs varied between 15 and 24

indicating a distinctly progressed karyotypic evolution. The most frequent CNAs were gains of the entire chromosome 6 or, at least, parts of it, and of 7p22. Other changes were gains of 3q26qter and the entire chromosome 7 and losses of segments on chromosome 4 q and of the short arm of chromosome 10. Enh(3q21q25), dim(4q22q33) and dim(4qter), dim(9p21), dim(13q22), enh(15-q14), and enh(18q22q23) were also frequently observed. Using a hierarchical cluster analysis, the specific patterns of genomic imbalance allowed the separation into two main groups indicating different karyotypic evolutions.

As a next step, we applied CGH to clinical biopsies of tumors to determine whether meaningful data can be obtained which are predictive for response to therapy and survival time. We and others applied CGH on oral squamous cell carcinoma [37]. Gain of 11q13 was involved in advanced stages of malignancy in oral squamous cell carcinoma. In addition, the proportion of patients deceased within one year after diagnosis was higher in the group whose tumors showed an increased 11q13 copy number as compared to the group without this increase. This could point to an association of gain in 11q13 and tumor aggressiveness [38]. Several cancer-related genes reside to this chromosomal locus making the association with tumor aggressiveness and an enhancement of band 13 at the long arm of chromosome 11 reasonable. Among these genes are the oncogenes *EMS1, EMSY, FGF3, FGF4*, the cell-cycle regulator *CCND1* and the *TAOS1* gene, which is involved in tumor progression. This locus also harbors the *ORAOV1* gene (oral cancer overexpressed gene 1). Patients with tumors characterized by the gain of 3q26-qter plus 5p14–p15 died earlier (i.e., less than 15 months) after excision of the tumor compared to the group without these imbalances [39]. Nineteen of 35 tumors showed a gain of chromosome band 7p12 [239], where the gene for the epidermal growth factor receptor (*EGFR*) is located. A highly complex but strikingly consistent pattern of other genomic imbalances (average, 32 CNAs per tumor) was associated with the 7p12 alteration. Average disease-free survival of tumors without a 7 p gain clearly exceeded that of tumors with a gain of 7 p (36.8 vs. 21.3). Relapse occurred in 63% in the 7p12-positive vs. 25% in the negative group. Average disease-free survival of tumors without the 7 p gain clearly exceeded that of tumors with the gain of 7 p (36.8 vs. 21.3). Then, genomic imbalances were investigated by hierarchical cluster analysis and clustered image mapping to investigate whether genomic profiles correlate with clinical data. There was indeed a significant relationship: patients suffering from tumors without enh(7p12) lived significantly longer than patients with tumors that harbor enh(7 p) ($p = 0.024$) [39,40]. The data clearly show that several genomic imbalances may affect the clinical outcome in human oral squamous cell carcinoma.

DNA methylation: The genome-wide simultaneous methylation status of CpG islands in colorectal carcinoma was investigated by means of a microarray-based technique [31]. Amplicons from tumor and control samples were pools of differentially methylated CpG island fragments hybridized to a panel of approximately 8000 CpG island tags. Data analysis identified 694 CpG island loci hypermethylated in a group of 14 colorectal tumors. The Stanford hierarchical cluster algorithm segregated the tumors into two subgroups, one of which exhibited a high level of concurrent hypermethylation while the other had little or no methylation. This is in agreement with observations of a CpG island methylation phenotype present in colorectal tumors [42]. The present study demonstrates that this microarray-based technique is useful in classifying tumors according to their methylation profiles.

Single nucleotide polymophisms: An important result of the human genome project is the high DNA variability. Statistically a genetic variation (polymorphism) occurs once in 1200 bases. In most cases, polymorphic gene variants lead to a diminished protein function; in some cases, however, increased activities have been reported [1]. In contrast to somatic mutations, i.e., in cancer, polymorphisms in germ line cells are stable and heritable. Polymorphisms include single nucleotide polymorphisms (SNPs), and length differences in micro- and minisatellites. An SNP represents a single base exchange that may or may not cause an amino acid exchange in the encoded protein. The frequency of SNPs is greater than 1% in a population and accounts for over 90% of genetic variation in the human genome. The number of SNPs has been estimated in a range from 1 to 10 million [43–45]. Between 50000 and 250000 SNPs are distributed in and around coding genes [46].

In our research, we focused on SNPs in drug transporter genes. The ATP-binding cassette (ABC) transporter family consists of 49 members. More than 10 of them are implicated in drug resistance to cancer chemotherapy [47–49]. They are important determinants of drug absorption, tissue targeting, and drug elimination. ABC transporters confer drug resistance by lowering the intracellular drug concentrations down to sublethal levels. Cancer cells, which express P-glycoprotein (*ABCB1, MDR1*) reveal a multidrug resistance phenotype to a broad range of structurally and functionally different drugs, including anthracyclines, anthracenediones, *Vinca* alkaloids, taxanes, epipodophyllotoxins, and others. P-glycoprotein is also expressed in various normal organs, such as brain vessels, adrenal gland, kidney, liver, and gastrointestinal tract. P-glycoprotein contributes to the blood-brain barrier, translocates hormones, and detoxifies xenobiotics taken up along with nutrients. Although the clinical therapy failure of tumors is multifaceted, its role for drug resistance is evident, and the prognostic significance of P-glycoprotein as an indicator for failure of chemotherapy and poorer outcome has been demonstrated in a number of studies [50–55]. As of yet, 29 polymorphisms have been identified in the *ABCB1* gene, with considerable differences in their frequencies among ethnic groups [56–59]. Of them, the G2677T/A SNP in exon 21 and the C3435T SNP in exon 26 have been most intensively studied, because they diminish the expression and function of P-glycoprotein [58,60]. Whereas the C2677T/A polymorphism causes an A893S/T amino acid substitution, the functional relevance of the C3435T variant is unknown, because this is a silent SNP. It is possible that specific haplotypes of the *ABCB1* (*MDR1*) gene might determine the efficacy and toxicity of drugs. The effect of the G2677T and C3435T SNPs on the pharmacokinetics and pharmacodynamics of drugs is still a matter of controversy, since contrasting results have been provided [61–68]. The expression of P-glycoprotein in normal tissues is thought to play an important role for the pharmacokinetics and pharmacodynamics of many drugs of different drug classes. A definitive answer on the role of *ABCB1* SNPs requires further studies.

Predictive mRNA markers

We have developed a low-density DNA microarray which contains 38 genes of the ATP-binding cassette (ABC) transporter gene family [69]. This tool has been validated with three different multidrug-resistant sublines (CEM/ADR5000, HL60/AR, and MCF7/CH1000) known to overexpress either the *ABCB1 (MDR1), ABCC1*

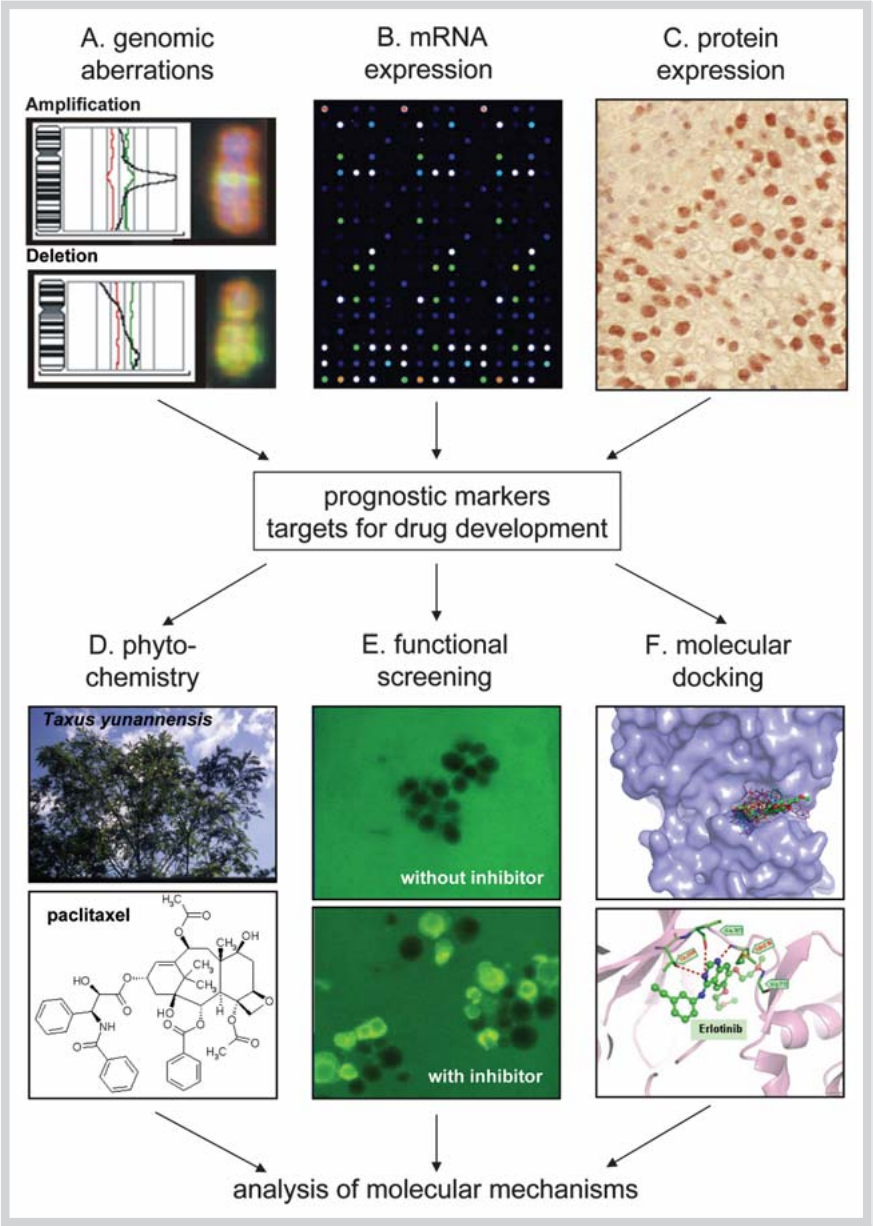

Fig. 1 Combining molecular diagnostics and natural product research for personalized cancer medicine. Biomarkers can be identified at the DNA, mRNA, and protein levels. Markers with prognostic significance for response to treatment and patients' survival can serve as a target for the development of novel drugs. Natural products with activity towards cancer cells can be identified by phytochemistry. Functional screening and molecular docking allow screening for inhibitors of drug targets. The genomic aberrations in (**A**) show an amplification of 5q13q14 harboring the *DHFR* gene in methotrexate-resistant CEM/MTXR3 cells. The deletion represents the loss of chromosomal locus 9p21 cells in CCRF-CEM cells, where the *MTAP* gene and the tumor suppressor genes *INK4A*, *INK4B*, and *ARF* are located [34]. **B** The microarray hybridization shows the mRNA expression in a breast cancer biopsy [146]. **C** Immunohistochemical staining of the oncogene product, c-MYC, in a lung cancer biopsy [147]. C-MYC is specifically localized in the nuclei of the cells. **D** Several yew species, including the Chinese *Taxus yunannensis* contain paclitaxel, which is already an established anticancer drug. **E** Functional screening for inhibitors of P-glycoprotein can be done by using fluorescence microscopy and rhodamine 123 (R123) as a fluorescent probe. The cytotoxic R123 is extruded by multidrug-resistant cells (black cells). Upon treatment with a P-glycoprotein inhibitor, cells start to take up R123 and die. Shown are P-glycoprotein-expressing leukemia cells from a patient, which were treated with R123 and without and with the P-glycoprotein inhibitor chloropromazine. **F** Molecular docking of drug molecules to three-dimensional crystal structures allow the *in silico* screening for putative inhibitors of therapeutic targets. Shown is the docking of natural products derived from traditional Chinese medicine to the tyrosine kinase binding domain of the human epidermal growth factor receptor (top) and the amino acids located in this binding pocket responsible for binding of erlotinib, a well-known EGFR-inhibitor. Erlotinib was used as a control drug [40]. Parts of the figure were taken from the above-cited references with permission of the publishers.

(MRP1), or *ABCG2 (MXR, BCRP)* genes. When compared with their drug-sensitive parental lines, we observed not only the overexpression of these genes in the multidrug-resistant cell lines but also of other ABC transporter genes, pointing to their possible role in multidrug resistance. These results were corroborated by quantitative real-time reverse transcription-PCR [69].

As a next step, we applied this microarray to detect drug resistance in clinical samples. We identified four new ABC transporters, which were overexpressed in many samples of patients with acute myeloid leukemia (AML) compared with healthy bone marrow: *ABCA2, ABCA3, ABCB2*, and *ABCC10* [70]. The overexpression of these four genes was verified by real-time PCR in 42 samples from children with AML and 18 samples of healthy bone marrow. The median expression of *ABCA3* was three times higher in 21 patients who had failed to achieve remission after the first course of chemotherapy than in a well-matched group of 21 patients who had achieved remission at this stage ($p = 0.023$). Incubation of cell lines with a number of different cytostatic drugs induced an upregulation of *ABCA3*. Downregulation of *ABCA3* by small interfering RNA sensitized cells to doxorubicin. These results show that *ABCA2, ABCA3, ABCB2*, and *ABCC10* might play a role for AML. ABCA3 is the most likely transporter to cause drug resistance.

Furthermore, we observed a consistent overexpression of *ABCA2/ABCA3* in clinical samples of T-cell acute lymphoblastic leukemia (T-ALL) [71]. Therefore, we analyzed the association of these two genes with drug resistance. Treatment of CCRF-CEM and Jurkat cells with methotrexate, vinblastine, or doxorubicin led to an induction of *ABCA3* expression, whereas a significant increase of *ABCA2* expression was only observed in Jurkat cells. To study the causal relationship of *ABCA2/A3* overexpression with drug resistance, we applied RNA interference (RNAi) technology. RNAi specific for *ABCA2* or *ABCA3* led to a partial decrease of expression in these two ABC transporters. Upon cotreatment of RNAi for *ABCA2* with methotrexate and vinblastine, a partial decrease of *ABCA2* expression as well as a simultaneous increase of *ABCA3* expression was observed. *Vice versa, ABCA3* RNAi plus drugs decreased *ABCA3* and increased *ABCA2* expression. This indicates that downregulation of one ABC transporter was compensated by

the upregulation of the other. Application of RNAi for both *ABCA2* and *ABCA3* resulted in a more efficient reduction of the expression of both transporters. As a consequence, a significant sensitization of cells to cytostatic drugs was achieved. In conclusion, *ABCA2* and *ABCA3* are expressed in many T-ALL and contribute to drug resistance.

Predictive protein markers

So far, our data obtained from CGH and DNA methylation analysis indicate that no single mechanism can explain the resistance to chemotherapy. The multifactorial nature of drug resistance implies that the analysis of comprising expression profiles may predict drug resistance with higher accuracy than single gene or protein expression studies. Therefore, 40 cellular parameters (drug resistance proteins, proliferative, apoptotic, and angiogenic factors, products of proto-oncogenes, and suppressor genes) were evaluated mainly by immunohistochemistry in specimens of primary non-small cell lung carcinoma (NSCLC) of 94 patients and compared with the response of the tumors to doxorubicin *in vitro* [72]. The protein expression profile of NSCLC was determined by hierarchical cluster analysis and clustered image mapping. The cluster analysis revealed three different resistance profiles. The frequency of each profile was different. The resistance proteins P-glycoprotein (*MDR1, ABCB1*), thymidylate synthetase, glutathione-*S*-transferase-π, metallothionein, O^6-methylguanine-DNA-methyltransferase and major vault protein/lung resistance-related protein were most frequently upregulated in one of the three clusters, while microvessel density, the angiogenic factor vascular endothelial growth factor and its receptor FLT1, and ECGF1 as well were downregulated. In addition, the proliferative factors proliferating cell nuclear antigen and cyclin A were reduced compared to the sensitive NSCLC. In this resistance profile, FOS was upregulated and NM23 downregulated. In the second profile, only three resistance proteins were increased (glutathione-*S*-transferase-π, O^6-methylguanine-DNA methyltransferase, major vault protein/lung resistance-related protein). The angiogenic factors were reduced. In the third profile, only five of the resistance factors were increased (P-glycoprotein, thymidylate synthetase, glutathione-*S*-transferase-π, O^6-methylguanine-DNA-methyltransferase, major vault protein/lung resistance-related protein).

In a second analysis, we analyzed the expression of resistance-related proteins for survival times of patients. NSCLC is usually associated with a poor survival prognosis [73]. Some patients survive their disease, and the underlying molecular mechanisms are still poorly understood. Therefore, we have evaluated the expression of 21 gene products (oncogene and tumor suppressor products and proliferative, apoptotic, and angiogenic factors) in paraffin-embedded primary NSCLCs from 216 patients and correlated the data with the survival times of the patients (survival of more or less than five years). The protein expression of FOS, P53, RAS, ERBB1, JUN, PCNA, cyclin A, FAS/CD95, and HIF-1β revealed a correlation to survival by means of the χ^2 test. In a second step, these nine parameters were further analyzed by hierarchical cluster analyses of all patients, of stage III patients, and of patients with squamous cell lung carcinomas. We identified clusters with significantly more long-term survivors. The expression of FOS, JUN, ERBB1, and cyclin A or PCNA were decreased in carcinomas of patients with long-term survival. The expression profile of these factors predicts a significantly better long-term outcome of NSCLC patients. This may have implications for the development of individualized therapy options in the future.

Survival time of patients is mostly influenced by metastasis. Therefore, we were also interested to investigate the role of these proteins for metastasis and to evaluate whether different protein expression patterns exist in primary squamous cell lung carcinomas of patients with and without lymph node involvement. Formalin-fixed, paraffin-embedded specimens from 130 patients with squamous cell lung carcinomas were analyzed by immunohistochemistry [74]. In a first step, proteins were selected which showed a relationship to lymph node involvement. The expression of JUN, ERBB2, MYC, cyclin D, PCNA, bFGF, VEGF and Hsp70 proteins revealed a positive correlation to lymph node involvement. In contrast, caspase-3, Fas ligand, Fas/CD95, and PAI showed an inverse correlation to lymph node involvement. In a second step, these parameters were further analyzed by hierarchical cluster analyses. The resulting clusters were correlated to patients with or without lymph node involvement. The data show that different protein expression patterns exist between primary squamous cell lung carcinomas with and without lymph node involvement and within carcinomas with lymph node involvement. The data suggest that various metastasis profiles exist. This has also been shown for other tumor types by other authors [75–83].

Molecular Targeted Therapy with Natural Products

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A chemical "treasure box" from medicinal plants

Given the fact that a considerable portion of all drugs used nowadays in oncology as well as in general pharmacology are from natural origin [12,13], natural products represent a valuable source for drug development. Surprisingly, this potential is frequently underestimated in pharmacology, biology, and medicine. In Europe, medicinal herbs gradually lost importance in the course of chemistry's progress in industrialized countries during the 20th century. From the perspective of the pharmaceutical industry, chemical compounds from natural origin may pose more problems concerning intellectual property and patenting issues. Furthermore, plant extracts can hardly be subjected to high-throughput screening technologies. For pharmaceutical chemists, it might be more attractive to fiddle about huge chemical libraries of synthetic compounds obtained by combinatorial chemistry than to isolate compounds from plants, which can be painful and time-consuming. This might at least in part explain the decline of natural products in drug development during the 20th century. Fortunately, the potential of natural products for chemotherapy seems to be rediscovered very recently, and the current thriving revival of phytotherapy is followed by an increasing scientific interest in bioactive compounds as lead drugs for semi-synthetic modification.

Our own interest to identify novel cytotoxic compounds with activity against tumor cells derived from medicinal plants used in traditional Chinese medicine (TCM) was raised in the 1990s [84]. The rationale behind this approach is that TCM looks back on a tradition of more than 5000 years. Hence, it can be expected that many ineffective medicinal herbs have vanished over the centuries. Indeed, a number of clinical studies were conducted on TCM providing convincing evidence to gain credibility and reputation outside China. As recently reviewed, clinical trials with TCM remedies focus on three major fields in cancer research: (i) improvement of poor treatment response rates towards standard chemo- and radiotherapy, (ii) reduction of severe

adverse effects of standard cancer therapy, and (iii) unwanted interactions of standard therapy with herbal medicines [85].

Apart from the approved drugs artemisinin and its semisynthetic derivative artesunate [86,87], we have analyzed cellular and molecular mechanisms of several other chemically characterized natural products derived from TCM. Among them were known compounds with still insufficiently defined modes of action, which were investigated by us using molecular biological and pharmacogenomic approaches, i.e., arsenic trioxide, ascaridol, berberine, cantharidin, cephalotaxine, curcumin, homoharringtonine, luteolin, isoscopoletin, scopoletin, vitexin, isovitexin, and others [88–109]. Furthermore, several novel bioactive compounds were described and analyzed in the course of our investigations, i.e., tetracentronsine A, tetracentronsides A, B and C, the two novel α-tetralone derivatives, berchemiasides A and B, as well as the novel flavonoid quercetin-3-*O*-(2-acetyl-α-L-arabinofuranoside) [110–112]. Artemisinin also reveals profound antimalarial activity [14]. During the past years, several derivatives (artesunate, artemether, arteether, artelinate) have been synthesized to improve the antimalarial activity.

Furthermore, we started the analysis of antiviral effects of natural products. We were the first to demonstrate that artesunate inhibits its NF-κB activity, leading to the inhibition of viral replication. NF-κB is involved in the transcriptional regulation of immediate early, early, and late proteins of human cytomegalovirus (HCMV) necessary for viral replication [113]. Artesunate also acts against cytomegaloviruses *in vivo* [114]. The antiviral activity of artesunate is not limited to HCMV. We showed that herpes simplex virus 1, hepatitis B and C viruses and others are also efficiently inhibited by artemisinin and artesunate [115,116].

Summarizing the results of our investigations in search of molecular markers, it turned out that patient survival and response to chemotherapy is multifactorial and that no single factor sufficiently explains treatment failure and death. We conclude that resistance markers may also be valuable targets for strategies to develop targeted therapies for individual cancer patients. The idea is to identify small molecules, which inhibit proteins that are essential for therapy response and worse prognosis. The focus of our own research is on small molecules from natural origin. We have selected three targets to prove the validity of this concept: (i) the chromosomal locus 9p21 harbors several genes, including *MTAP, INK4A, INK4B*, and *ARF. MTAP* offers the opportunity for a chemoselective treatment which affects tumor cells with 9p21 deletion but spares normal tissues; (ii) P-glycoprotein is an efflux transporter that extrudes many anticancer drugs out of tumor cells rendering them resistant to chemotherapy. We have searched for compounds that are not recognized by P-glycoprotein and which, hence, kill P-glycoprotein-expressing multidrug-resistant tumor cells with a similar efficacy than P-glycoprotein-negative drug-sensitive cells. Alternatively, the inhibition of this drug transporter by small molecules may overcome multidrug resistance; (iii) EGFR represents an important signal transducing molecule regulating tumor growth, apoptosis, differentiation and other key processes. The recognition of its value as a target for novel drugs resulted in the development of therapeutic antibodies and small molecules. However, tumor cells can also exert resistance to these novel therapeutics and novel EGFR inhibitors are required.

Chemoselective treatment of tumor cells with 9p21 deletion by L-alanosine

Unfortunately, most established anticancer drugs not only kill tumor cells but also affect normal tissues [117]. It would, thus, be desirable to have treatment targets which allow the distinction between normal and cancerous tissue.

A deletion of the short arm of chromosome 9 at band 21 (9p21) is a frequent chromosomal aberration in many tumor types including acute lymphoblastic leukemia [118,119]. This locus harbors the tumor suppressor genes $p16^{INK4A}$, its alternative splice product $p14^{ARF}$, and $p15^{INK4B}$. Their gene products regulate the progression from G1 to S phase of the cell cycle via the RB1 or p53 pathways [120]. In addition, the methylthioadenosine phosphorylase (*MTAP*) gene is also localized at this chromosomal region and $p16^{INK4A}$ and *MTAP* genes are frequently codeleted in tumors [121]. MTAP converts methylthioadenosine into adenine and 5-methylthioribose 1-phosphate by phosphorolysis. Adenosine is used to recruit adenine nucleotide pools for DNA synthesis. Methylthioadenosine can, therefore, serve as an alternative purine source, if the *de novo* purine biosynthesis is inhibited by antimetabolites, i.e., methotrexate [122]. Tumor cells with a deletion of the *MTAP* gene at chromosome 9p21 cannot use this salvage pathway and die upon methotrexate challenge. As all normal tissues have MTAP activity [122], they do not die at methotrexate concentrations lethal for MTAP-deficient cancer cells. The MTAP salvage pathway may, thus, offer a unique opportunity for a selective tumor therapy which spares normal tissues. This treatment advantage may, however, vanish in MTAP-deficient cells, which developed resistance towards MTX, i.e., by amplification of the dihydrofolate reductase gene.

Therefore, we analyzed the role of MTAP for chemoselectivity of the antimetabolites trimetrexate and L-alanosine and whether cross-resistance to methotrexate hampers their effectiveness [123,124]. Trimextrexate is a derivative of the established anticancer drug methotrexate. L-Alanosine is an amino acid analogue and antibiotic derived from the bacterium *Streptomyces alanosinicus*. The analyses were performed with CCRF-CEM cells in which the 9p21 deletion was found by CGH and fluorescence *in situ* hybridization. T-cell acute lymphoblastic leukemia (T-ALL) cells (CCRF-CEM) were transfected with an *MTAP* expression vector. A green fluorescent protein (*GFP*) plasmid was co-transfected to monitor the transfection efficacy by flow cytometry. The response of *MTAP*-transfected cells to the antimetabolites methotrexate, trimetrexate, and L-alanosine was decreased compared to mock control transfectants using growth inhibition assays. The activity of doxorubicin which is not involved in DNA biosynthesis was not changed in *MTAP* transfectants. As the $p16^{INK4A}$ tumor suppressor gene resides also at 9p21, we transfected CCRF-CEM cells with a $p16^{INK4A}$ expression vector. These transfectant cells were more resistant to all four drugs indicating that $p16^{INK4A}$ did not specifically affect antimetabolites. The chemoselective effect of antimetabolites in *MTAP*-deleted tumor cells may, however, be compensated by the development of drug resistance. To prove this possibility, we analyzed a methotrexate-resistant subline, CEM/MTX1500LV, in which the methotrexate resistance-conferring dihydrofolate reductase (*DHFR*) gene was amplified. While trimetrexate exhibited considerable cross-resistance in CEM/MTX1500LV cells, L-alanosine did not. Thus, L-alanosine could exhibit chemoselectivity in 9p21/MTAP-deleted cells, even if *DHFR* amplification occurs. We conclude that L-alanosine may be more suitable than methotrexate or trimetrexate for *MTAP*-mediated chemoselective treatment of T-ALL. Pre-ther-

apeutic detection of 9p21 and *MTAP* deletion may be helpful in developing a predictive molecular chemosensitivity test for T-ALL.

Natural products that bypass or modulate P-glycoprotein-mediated multidrug resistance

Non-cross-resistant natural products: In a recent investigation, we analyzed the cross-resistance profile of clinical samples of 59 tumors of different origins and 38 lung tumors *in vitro* [125]. Cytostatic drugs from different classes were used (anthracyclines, antibiotics, *Vinca* alkaloids, epipodophyllotoxins, antimetabolites, and alkylating agents). Tumors exert broad resistance profiles. Tumors resistant to one drug also tend to be resistant to other drugs, while sensitive tumors reveal sensitivity towards many drugs. Expression of P-glycoprotein and the proliferative activity of tumors were identified as underlying mechanisms of broad spectrum resistance.

As a second step, the cross-resistance profiles were analyzed in sensitive and resistant cell lines from different tumor types to study underlying mechanisms. In an effort to find new treatment possibilities, novel cytotoxic compounds with activity against otherwise drug-resistant tumor cells were investigated [125]. We used the CEM/ADR5000 leukemia cell line overexpressing P-glycoprotein/*MDR1* and its parental cell line, CCRF-CEM to investigate cross-resistance profiles. CEM/ADR5000 cells were more than 1000-fold resistant to the selecting agent, doxorubicin. They were also highly cross-resistant to the anthracycline epirubicin (484-fold) but less cross-resistant to idarubicin (6.9-fold). We also tested the anthracycline metabolites doxorubicinol, epirubicinol, and idarubicinol, which revealed cross-resistance to a lesser degree than the non-metabolized parental drugs (range from 1.6- to 382-fold). Cross-resistance to the *Vinca* alkaloids vincristine, vinblastine, vindesine, and vinorelbine was in a range of 14- to 613-fold and to the taxanes paclitaxel and docetaxel in a range of 200- to 438-fold. The degrees of resistance to the epipodophyllotoxines etoposide and etoposide phosphate were lower (18- and 11-fold, respectively). In an effort to identify novel compounds with activity against otherwise drug-resistant tumor cells, we analyzed natural products derived from medicinal plants used in traditional Chinese medicine (TCM) in CCRF-CEM and CEM/ADR5000 cells. Interestingly the multidrug-resistant cells revealed either low degree of cross-resistance (cephalotaxine, berberine, homoharringtonine, maesopsin), no clear cross-resistance (*E*)-3-(4-hydroxyphenyl)-[2-(4-hydroxyphenyl)-ethyl]-prop-2-enamide, *N*-*p*-coumaryl tyramine, maslinic acid) or even enhanced sensitivity towards the natural products from TCM [cantharidin, tetracentronside, 3-(2-hydroxyethyl)-1*H*-indole-5-*O*-β-D-glucopyranoside, kaempferol, artesunate].

In order to gain a systematic approach in identifying natural products from TCM with inhibitory activity against multidrug-resistant tumor cells, we compiled 531 cytotoxic natural products and derivatives thereof in a database [126]. These compounds were tested in the drug screening program of NCI (www.dtp.nci.nih.gov). In combination with microarray data, the generation of hypotheses regarding their modes of action is a starting point for further mechanistic studies. We correlated the IC_{50} values of the 60 NCI tumor cell lines for these 531 natural products with the accumulation data for rhodamine 123 (R123) as a functional assay for P-glycoprotein and with the cell doubling times as a parameter of proliferation. While the IC_{50} values for only 18 compounds correlated with R123 accumulation (3%), 162 natural products were significantly associated with the cell doubling times of the cell lines (31%), indicating that natural products might be a rich source for novel drug candidates with activity to bypass P-glycoprotein- and proliferation-associated drug resistance. We will systematically exploit the chances to develop novel cancer drugs from TCM with improved features.

Modulators of P-glycoprotein function: Another concept was the blockage of P-glycoprotein by specific inhibitors. Despite huge efforts in academia and industry, no P-glycoprotein inhibitor has clinically showed satisfying results and reached the pharmaceutical market yet [127, 128]. Most of these resistance-modifying agents (RMA) are too toxic at the required doses. Therefore, the development of novel RMAs to overcome MDR represents a major challenge to modern cancer chemotherapy. In this context, we analyzed natural products for their ability to inhibit P-glycoprotein function.

The antimycobacterial quinolones 1-methyl-2-undecyl-4-quinolone, dihydroevocarpine and evocarpine as well as the indoloquinazoline alkaloids rutaecarpine and evodiamine – all from the Chinese medicinal herb *Evodia rutaecarpa* – were tested in two *in vitro* assays, for cytotoxicity and interaction with P-glycoprotein. Cytotoxicity was measured in a cell proliferation assay against sensitive CCRF-CEM and multidrug-resistant CEM/ADR5000 cells. An assay monitoring the P-glycoprotein-dependent accumulation of the dye calcein in porcine brain capillary endothelial cells (PBCECs) was used to study interactions of the test substances with this efflux pump. Rutaecarpine and evodiamine showed quite a high toxicity with IC_{50} values from 2.64 to 4.53 µM and were weak modulators of P-glycoprotein activity. The degrees of resistance in CEM/ADR5000 towards the saturated quinolones 1-methyl-2-undecyl-4-quinolone and dihydroevocarpine were between 3 and 4. In the calcein assay, these two quinolones were shown to be modulators of P-glycoprotein activity. Evocarpine, on the other side, is not transported by P-glycoprotein, and showed only slight toxicity at the highest test concentration of 30 µM [129]. Furthermore, four antimycobacterial geranylated furocoumarins, from the fruits of *Tetradium daniellii* (Rutaceae) showed considerable cell proliferation inhibition with IC_{50} values ranging from 1.72 to 11.02 µM against CCRF-CEM and 2.09 to 13.56 µM against CEM/ADR5000, respectively. The calcein assay to monitor P-glycoprotein function showed that all four compounds are modulators of P-glycoprotein [130].

In another project, we described the effect of oxalyl-bis(*N*-phenyl)hydroxamic acid (OBPHA) and copper *N*-(2-hydroxyacetophenone)glycinate (CuNG) on multidrug-resistant P-glycoprotein-expressing CEM/ADR5000 T-cell acute lymphoblastic leukemia cells [131]. CuNG, a known depleting agent for glutathione (GSH) and inhibitor of glutathione *S*-transferase (GST) and multidrug resistance-related protein 1 (MRP1), also inhibited P-glycoprotein-mediated doxorubicin accumulation and retention. The resistance-modifying effects of OBPHA were stronger than that of CuNG. Both novel RMAs overcame drug resistance more efficiently than verapamil, a well-known P-glycoprotein inhibitor. OBPHA and CuNG exposure resulted in an increased doxorubicin accumulation after 1–3 h incubation by downregulation of P-glycoprotein expression after 24 h incubation. This is a clue that different mechanisms may contribute to modulation of P-glycoprotein-mediated drug resistance by these compounds.

We investigated the effect of CuNG on reactive oxygen species (ROS) generation and antioxidant enzymes in normal and doxorubicin-resistant Ehrlich ascites carcinoma (EAC/Dox)-bearing Swiss albino mice [132]. The effect of CuNG has been studied on ROS generation, multidrug resistance-associated protein1

(MRP1) expression and on activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). CuNG increased ROS generation and reduced MRP1 expression in EAC/Dox cells while only temporarily depleted glutathione (GSH) within 2 h in heart, kidney, liver and lung of EAC/Dox-bearing mice, which were restored within 24 h. The level of liver copper was observed to be inversely proportional to the level of GSH. Moreover, CuNG modulated SOD, CAT and GPx in different organs and thereby reduced oxidative stress. Hence, nontoxic doses of CuNG may be utilized to reduce MRP1 expression and thus sensitize EAC/Dox cells to standard chemotherapy. Moreover, CuNG modulated SOD, CAT and GPx activities to reduce oxidative stress in some vital organs of EAC/Dox-bearing mice. CuNG treatment also helped to recover liver and renal function in EAC/Dox-bearing mice.

Furthermore, we have determined the efficacy of CuNG in overcoming multidrug-resistant cancer using drug-resistant murine and human cancer cell lines [133]. The action of CuNG following single *i.m.* administration (5 mg/kg body weight) was tested *in vivo* on doxorubicin-resistant Ehrlich ascites carcinoma (EAC/Dox)-bearing mice and doxorubicin-resistant sarcoma 180-bearing mice. Tumor size, ascitic load, and survival rates were monitored at regular intervals. Apoptosis of cancer cells was determined by cell cycle analysis, confocal microscopy, annexin V binding, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay *ex vivo*. IFN-γ and tumor necrosis factor-α were assayed in the culture supernatants of *in vivo* and *in vitro* CuNG-treated splenic mononuclear cells from EAC/Dox-bearing mice and their apoptogenic effect was determined. Sources of IFN-γ and changes in the number of T regulatory marker-bearing cells in the tumor site following CuNG treatment were investigated by flow cytometry. Supernatants of *in vitro* CuNG-treated cultures of peripheral blood mononuclear cells from different drug-insensitive cancer patients were tested for presence of the apoptogenic cytokine IFN-γ and its involvement in induction of apoptosis of doxorubicin-resistant CEM/ADR5000 cells. CuNG treatment could resolve drug-resistant cancers through induction of apoptogenic cytokines, such as IFN-γ and/or tumor necrosis factor-α, from splenic mononuclear cells or patient peripheral blood mononuclear cells and reduced the number of T regulatory marker-bearing cells while increasing infiltration of IFN-γ-producing T cells in the ascetic tumor site. Our results show the potential usefulness of CuNG in immunotherapy of drug-resistant cancers irrespective of multidrug resistance phenotype.

Besides P-glycoprotein, other drug transporters have also been described in recent years to confer drug resistance. Among them is the breast cancer resistance protein (BCRP, ABCG2). We have focused on this protein in the context of the highly active antiretroviral therapy (HAART), whose safety and effectiveness is frequently challenged by viral resistance to antiretrovirals and the frequent occurrence of drug interactions which may limit the access of these drugs to the target sites. In particular, drug distribution and elimination may be modified by BCRP. Therefore, we investigated the influence of all important anti-HIV drugs on BCRP activity *in vitro* in one assay to allow unrestricted comparison of the results [134]. BCRP inhibition was assessed by an increase in pheophorbide A accumulation in expressing MDCKII cells and compared with the corresponding parental cell line MDCKII lacking human BCRP. According to the IC_{50} estimation, the rank order for BCRP inhibition was lopinavir > nelfinavir > delavirdine > efavirenz > saquinavir > atazanavir > amprenavir > abacavir. Whereas nevirapine and zidovudine exerted weak inhibition, the inhibitory potency for ritonavir and tipranavir could not be estimated due to their low solubility and all other tested compounds (indinavir, didanosine, emtricitabine, lamivudine, stavudine, tenofovir and zalcitabine) were devoid of an effect. This study demonstrated a significant inhibition of BCRP by many anti-HIV drugs. This suggests that inhibition of BCRP might contribute to drug-drug interactions observed during HAART *in vivo* and possibly also the superior effectiveness of combination antiretroviral therapy.

Natural products that inhibit the epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) has an extraordinary relevance in tumor biology. In our CGH analyses with oral squamous cell carcinoma, the amplification of the locus 7p12 also points in this direction [39]. A number of novel treatment options for tumors overexpressing EGFR have been launched to the market in recent years, e.g., therapeutic antibodies and small molecules such as gefitinib and erlotinib. These two compounds compete with ATP for binding to the ATP-binding domain of EGFR and, thereby, kill cancer cells. Despite considerable success with EGFR inhibitors, resistance against these new treatment modalities can also occur [135] and novel EGFR tyrosine kinase inhibitors are urgently required.

Using the above-described database with 531 compounds derived from TCM [126]; 35 candidate compounds were identified by correlation analyses of their IC_{50} values and the microarray-based mRNA expression values of the EGFR gene in 60 cell lines of the NCI [40]. Among the 35 natural products, two have already been reported to be associated with EGFR function [136]. Then, molecular docking studies (or protein-ligand interaction studies) were carried out with these 35 compounds using the crystal structure of the EGFR tyrosine kinase domain as the docking template. As a control, two crystal structures of EFGR tyrosine-kinase domain with inhibitors (erlotinib and lapatinib, respectively) bound in the ATP-binding site were taken from the Protein Data Bank (www.rcsb.org). Visual inspection of erlotinib control docking studies showed that the predicted erlotinib binding site and its orientation in binding site agreed very well as observed in the crystal structure. The predicted K_i (pK_i) values in our approach also agreed well with the reported values in the literature for erlotinib ($K_i = 0.7 \pm 0.1$ nM) and gefitinib ($K_i = 0.4 \pm 0.1$ nM) [137].

The 35 selected TCM compounds were individually docked into the crystal structure of EGFR tyrosine-kinase domain (EGFR-TK) for the appropriate conformational search. Eighteen out of 35 natural products were docked in the erlotinib binding site with docked energy values in the range of −6.6 to −10.2 kcal/mol. Among them, neo-oxyberberine, dicentrine, piceatannol and organol scarlet exhibit similar binding features to that of erlotinib in the tyrosine-kinase active site of EGFR [40].

Binding site analysis showed that interaction of dicentrine, organol scarlet and erlotinib with residues Thr766 and Met769 was a conserved feature. Thr766 and Met769 formed a hydrogen bond with the quinazoline nitrogen atom of erlotinib, while they were involved in hydrogen bond formation with the benzodioxoloquinoline oxygen atom of dicentrine. In the case of organol scarlet, Thr766 and Met769 formed a hydrogen bond with the diazenyl nitrogen atom of organol scarlet that is not a part of any cyclic system as found in erlotinib [40]. Further investigations are underway to analyze these findings in more detail.

Conclusions and Perspectives

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Our data indicate that a great diversity of molecular mechanisms is operative in clinical drug resistance. We showed that different resistant profiles exist within tumors of homogeneous histology. Thus, it is possible to identify novel subgroups of otherwise homogeneous tumor groups. These results are in accordance with a large body of evidence in the literature [138–144]. The systematic investigation of combinations of cellular factors in cancer clearly yields improved predictive information. Recently developed technologies for genome-, transcriptome- or proteome-wide analyses facilitates the simultaneous analyses of thousands of genes or proteins in a single experiment, raising expectations that it will revolutionize cancer diagnosis.

On the other hand, the results of our group as well as of other authors [145] indicate that a minimal set of about 10 to 50 factors may be sufficient and may bring more robust results than sets of thousands of factors. Therefore, it is reasonable to focus on a few relevant prognostic factors which may serve as drug targets to treat individual patients exhibiting these specific poor prognostic factors. We consider these results as one step further to the ultimate goal of prediction of drug response of each individual patient. We have exemplarily done this for three target proteins, i.e., MTAP, P-glycoprotein and EGFR and showed the principal feasibility of this concept.

In the long run, it has to be seen whether genomics and proteomics along with other "-omic" technologies will provide real hope or just another hype. It has to be taken cautiously, bearing in mind that the response to chemotherapy depends not only on intracellular, e.g., molecular and biological factors, but also on extracellular factors. The relevance of pharmacokinetics and dynamics must not be underestimated.

Generally, our knowledge on cancer biology and molecular factors with prognostic significance has exponentially grown over the past two decades delivering a wide array of proteins that may serve as target structures for drug development. This will for sure lead to a tremendous increase in novel compounds to be tested in the years to come, and small molecules will be a major player in this scenario.

Given the fact that a considerable portion of all drugs used nowadays is from natural origin, natural products represent a valuable source for drug development. Chemical compounds developed over millions of years during evolution bear a tremendous potential as novel drugs. The hope is that this potential will be utilized for the sake of the cancer patients.

We will face a shift from the established, rather unspecific and toxic anticancer drugs to new generations of targeted drugs with improved pharmacological features concerning tumor specificity and toxicity. At the moment it cannot be foreseen whether cancer patients will routinely be curable. Rather, cancer might turn from a life-threatening into a chronic disease, which needs life-long therapy. This raises concerns, and one might ask "who should pay?" The only way to reduce costs is to avoid overtreatment of patients. Personalized medicine will provide sophisticated diagnostic tools to adjust the right treatment for the right patient. As a consequence, it is to be hoped that treatment failure and unwanted side effects will more and more disappear and treatment efficacy will raise improving quality of life.

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