

Chemoresistance in Non-Small Cell Lung Cancer

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Abstract: The treatment of advanced non-small-cell lung cancer (NSCLC) is based on the combination of platinum and one of the following agents: taxanes, gemcitabine, vinorelbine or irinotecan. There are no significant differences in efficacy among these combinations suggesting that the maximum efficacy has been reached. In this review, we will consider the mechanisms of chemoresistance of the five groups of cytotoxic drugs commonly used in the treatment of advanced NSCLC as well as the clinical studies which have assessed the value of chemoresistance markers. Breast Cancer Related Protein (BRCP) expression has been related to irinotecan and cisplatin (CDDP) resistance. DNA repair capacity influences response to CDDP and ERCC1 gene stands out as a predictive marker of CDDP sensitivity. Preliminary studies indicate that high tubulin III and stathmin mRNA levels correlate with response to paclitaxel and vinorelbine and that high expression of class III tubulin by tumor cells assessed immunohistochemically in patients receiving a taxane-based regimen is associated with a poor response to chemotherapy, and a shorter progression-free survival. High expression levels of ribonucleotide reductase has also been related to response to gemcitabine. Uridine diphosphate glucuronosyltransferase isoform 1A1 (UGT1A1) genotype has been reported to be associated with time to progression and survival in patients treated with irinotecan. These data suggest that pharmacogenomic strategies may be used for developing customized chemotherapy in prospective studies. Adjuvant chemotherapy which had recently shown its usefulness in limited lung cancer represents another area of investigation for pharmacogenomic studies.

Key Words: Cis-platin, drug resistance, gemcitabine, irinotecan, non-small-cell lung cancer, pharmacogenomics, taxanes, vinorelbine.

INTRODUCTION

Epidemiologic estimates for the year 2000 showed that lung cancer is the most common cancer in the world both in terms of incidence (with 1.2 million new cases corresponding to 12,3% of the world total) and mortality (with 1.2 million deaths corresponding to 17,8% of the total [1]. Non-small-cell lung cancer (NSCLC) which includes the major histological subtypes, adenocarcinoma, squamous cell carcinoma, and large cell carcinoma accounts for 80% of all lung cancers. More than half of NSCLCs are advanced stage IIIB or IV at presentation, and patients with advanced NSCLC are candidates for systemic chemotherapy. Except for some patients with surgically resectable disease, the prognosis for patients with NSCLC is poor. Chemotherapy has been shown to provide survival and quality of life benefits for patients with advanced stage, unresectable NSCLC, but overall 2-year survival rates for this group of patients remains less than 20% [2]. The standard treatment for stage III unresectable disease (without pleural or pericardial effusion) is a combined-modality therapy with radiotherapy and chemotherapy [3]. In metastatic disease, treatment is based on the combination of cisplatin (CDDP) or carboplatin and one of the new active agents including: taxanes (paclitaxel and docetaxel), gemcitabine, vinorelbine

and irinotecan [2, 3]. These new regimens have produced superior therapeutic results compared with CDDP alone and older CDDP-based regimens with a survival advantage of 8 to 10 months [4]. Recent randomized studies indicate that there are no significant differences in efficacy among these combinations of CDDP with these new drugs although they have shown varying profiles of toxicity [5, 6]. These results suggest that a maximum of efficacy has been reached in the treatment of NSCLC with the current therapeutic strategy, which consists in treating patients without taking into account the biological characteristics of their tumors .

New approaches are necessary to improve outcome of unresectable and metastatic NSCLC. Recently, several publications showed that genome expression may predict response to drugs and patient outcome. Moreover, pharmacogenetics, the study of genes that influence drug activity and toxicity, appears as an interesting way in offering the possibility of tailoring therapy to the specific profile of individual patients and tumors. A pharmacogenetic approach can thus potentially increase response rates and survival outcome while decreasing toxicity and overall treatment costs.

In this paper, we will review drug resistance mechanisms, including multidrug resistance and mechanisms specific to five groups of cytotoxic drugs most commonly used in the treatment of NSCLC. In a second part, we will review the clinical studies which have assessed correlations between potential chemoresistance markers and patient outcome.

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MULTIDRUG RESISTANCE

In cell culture systems, multidrug resistance is a phenotype exhibited by tumor cells which, although exposed to only a single natural product type drug, display cross-resistance to an array of structurally unrelated compounds [7]. Four types of multidrug resistance mechanisms have been defined on the basis of the cellular targets involved, i.e. classical multidrug resistance (*MDR*), non-P-glycoprotein *MDR* (also called *MRP*), lung resistance-related protein (*LRP*) and breast cancer resistance protein (*BCRP*). The first two types of resistance are respectively conferred by two proteins, the 170 kDa P-glycoprotein (PgP, encoded by the *mdr1* gene) and the 190 kDa multidrug resistance protein (*MRP1*, encoded by the *mrp1* gene) which is the first member to have been described in the *MRP* family. Although PgP and *MRP* both belong to the ATP-binding cassette (ABC) superfamily of transport proteins and they are associated with reduced drug accumulation in resistant cells, they differ substantially in their structure, physiological function and possibly their mechanisms of action. PgP acts as a detoxifying agent by pumping toxins or xenobiotics, including anticancer agents, out of cells. A heterogeneous group of hydrophobic antitumor drugs derived from natural products, e.g., vincristine, etoposide, doxorubicin, irinotecan, are good substrates for PgP-mediated efflux.

However, overexpression of PgP seems to have only a minor role in lung cancer resistance in the clinic. Goldstein *et al.* reported that messenger RNA levels were only detected in five of 19 lung cancer tissues and that levels of expression were low [8]. Lai *et al.* investigated the role of the *mdr1* gene expression in lung cancer by performing RNA slot blot analysis in samples from a panel of 24 lung cancers and 10 corresponding non tumorous lung tissues [9]. Almost all the tumors and non tumorous lung tissues expressed low levels of *mdr1* RNA without correlation with prior therapy status of the patients nor clinical response to therapy [9]. Scagliotti *et al.* showed with an immunohistochemical technique that in fifteen among eighty-six NSCLC patients, more than 25% of their cells stained positive for PgP, but the heterogeneity of the expression ranged from rare scattered cells to a positive pattern for nearly all cells considered, without any relation with prognosis [10]. Shin *et al.* also found that multidrugresistance RNA expression was only detectable in three of 23 untreated NSCLCs [11]. Oka *et al.* evaluated 87 lung cancer surgical tissue samples for the levels of *mdr1* mRNA determined by Northern blotting [12]. Fifteen percent (13 out of 87) of the tumors were positive for the *mdr1* gene, but the level was low. The gene expression did not correlate with any pathological factor and was not associated with tumor progression nor drug resistance. Nevertheless, Brooks *et al.* showed by immunohistochemistry that low expression of *MDR* was a possible independent marker of improved cancer-free-survival in 59 patients treated with vinorelbine-based chemotherapy and radiotherapy for stage III disease [13].

Taken together these results indicate that classical multidrug resistance mediated by PgP is likely to have only a minor role in drug resistance in lung cancer.

MRP1 is an integral membrane phosphoglycoprotein encoded by a 6.5-kilobase mRNA, discovered in cell lines

which displayed multidrug resistance but did not overexpress PgP. As in the case of PgP, transfection of drug-sensitive cells with a full-length *MRP* cDNA is sufficient to confer resistance to doxorubicin, vincristine, etoposide, irinotecan and colchicine [14].

Investigations of the clinical relevance of *MRP* in lung cancer are still in their early phase. Sugawara *et al.* showed that *MRP* protein detected by immunohistochemistry was abundantly expressed in 28 of 59 adenocarcinoma specimens [15]. In contrast, *MRP* expression in other cancer types was lower (less than 20 % in squamous cell carcinomas and 20% in large-cell carcinomas) [15]. Ota *et al.* investigated the levels of expression of the *mrp* gene, quantified by Northern blot analysis, in comparison with those of *mdr1* in 104 NSCLC specimens (16). Thirty-three patients (32%) expressed the *MRP* gene at various levels. Sixty-one of the 104 NSCLC patients received postoperative chemotherapy with vindesine and etoposide. Twenty-three patients (38%) with tumors expressing high or moderate levels of *MRP* had significantly worse prognoses than those with non-or low-*MRP*-expressing tumors [16]. Wright *et al.* detected *MRP* in the majority of all histological subtypes of 112 NSCLCs [17]. No correlation was observed between *MRP* expression and any clinicopathological parameter [17]. In another study, Young *et al.* found that both *MRP1* and *MRP3*, but not *MDR*, *MRP2*, *MRP4*, *MRP5* protein levels were correlated with decreased sensitivity of lung cancer cell lines to doxorubicin, vincristine, VP-16 and CDDP [18].

A more recently cloned gene, the *lrp* gene, appears to be associated with a multidrug-resistance phenotype [19]. This gene codes for a 110 kDa protein termed the lung resistance-related protein, also designated as the Major Vault Protein (MVP), that is overexpressed in several non-PgP *MDR* cell lines of different origins. Rather than actively pumping out the cytotoxic drug at the cell membrane, this protein is primarily located on internal membranes and responsible for the uptake of cytoplasmic drug into cytoplasmic vesicles. The sequestered drug cannot cause DNA strand breaks and would be slowly extruded from the cell by exocytosis. Kitozono *et al.* demonstrated, using an *LRP*-induction system and *LRP*-specific ribozymes, that *LRP* is involved in resistance to doxorubicin, vincristine, VP-16 and taxol [20]. In a preliminary study, Dingerms *et al.* studied 36 NSCLC samples for *LRP* expression and, in addition, 17 lung cancer samples (10 NSCLC and 7 small-cell lung cancer) derived from patients treated with chemotherapy were analyzed in order to investigate *LRP* and *MRP* expression and the response to chemotherapy [21]. In NSCLC patients *LRP* expression was not a prognostic factor for survival and did not predict the response to chemotherapy. These authors confirmed these results in another study which found no relationship between response to chemotherapy and expression of *MRP* or *LRP* analyzed by immunohistochemical staining in 38 samples of NSCLC [22]. In another study *LRP* expression detected by immunohistochemistry was detected in 45% of 87 cases of untreated NSCLC and its expression was correlated with resistance to doxorubicin [23].

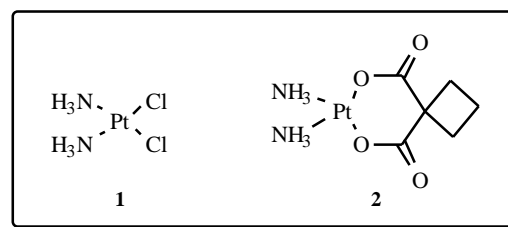
BCRP is another ABC half-transporter which was first isolated from human breast cancer MCF7/AdrVp-resistant

cell [24]. *BRCP* confers resistance against mitoxantrone, doxorubicin, and topoisomerase I (Topo I) inhibitors such as topotecan and irinotecan through the efflux of these drugs. *BRCP* appears to be an important mechanism of resistance against Topo I inhibitors, which have become key drugs for chemotherapy in lung cancers (25). Kawabata *et al* assessed the *BRCP* mRNA expression in 23 untreated NSCLC by real-time reverse transcription-PCR and found a widely variable expression in tumor tissues [25]. Five out of 23 expressed higher levels of *BRCP* mRNA than that found in NCI-H441 cells with active *BRCP* function conferring high resistance to topotecan suggesting a role in drug resistance *in vivo*. In a recently published study, Yoh *et al.* investigated the relationship between the level of expression of ABC transporter proteins examined immunohistochemically, and response to chemotherapy and prognosis in 72 untreated stage IIIB or IV NSCLC patients who had all received platinum-based chemotherapy [26]. Expression of PgP, *MRP1*, and *MRP3* was not significantly associated with response to chemotherapy or survival. In contrast the response rate to chemotherapy of patients with *BCRP*-negative tumors was 44%, as opposed to 24% in patients with *BCRP*-positive tumors (difference not statistically significant). *BRCP*-positive patients also had shorter progression-free survival ($p=0.0003$) and overall survival ($p=0.0004$) than *BRCP*-negative patients. Multivariate analysis including clinicopathological factors confirmed *BRCP* status as being an independent prognostic factor for survival [26]. These two studies suggest that *BRCP* expression levels may predict response to chemotherapy.

In a recent study, Szakacs *et al.* profiled mRNA expression of the 48 known human ABC transporters in 60 diverse cancer cell lines using real-time PCR [27]. By correlating the results with the growth inhibitory profiles of 1,429 anticancer drugs, they identified which transporters are more likely than others to confer resistance expression to which cytotoxics. They identified some compounds whose activity were enhanced, rather than antagonized, by the *MDR1* multidrug transporter. This gene expression database will be able to serve as a high-quality time capsule that can be mined to generate new hypotheses and to shed light on additional features of ABC transporters and their functional relationships with other molecules.

CISPATIN AND CARBOPLATIN

Cis-Diamminechloroplatinum (CDDP) “compound (1)” has a rigid structure with two labile chloro and two stable ammine ligands in a cis configuration. As in the case of other alkylating agents, the neutral drug molecule needs to be converted to a reactive form. This occurs non-enzymatically in solution, where displacement reactions result in stepwise exchange of the labile chloro ligands with water molecules. The charged aquated species are highly reactive, but the chloromonoaqua species is the most significant in the terms of interaction with DNA at physiological pH. In the case of the carboplatin “compound (2)”, which is a more stable bidentate cyclobutanedicarboxylate ligand, the aquation reaction is much slower. This reduces drug potency, which thereby requires a greater dose for an equivalent tumor effect [28]. Platinum exert their antineoplastic activity by binding covalently to DNA, thereby forming six different types of



adducts. The most abundant types of adducts are the intrastrand crosslinks between two adjacent bases (Pt-GG and Pt-AG adducts) which represent approximately 65 and 25% of the total number of adducts formed, respectively. Minor adducts are monofunctionally bound CDDP molecules to a guanine base and interstrand crosslinks between guanines on opposite strands.

The cellular mechanism of CDDP resistance has been revealed to be multifactorial and includes: 1) decreased platinum accumulation in the cytoplasm; 2) increased intracellular detoxification; 3) increased DNA repair capacity.

Reduced intracellular accumulation of CDDP which may arise because of decreased uptake or increased efflux is frequently observed in CDDP-resistant cell lines [29, 30]. To date the exact mechanism by which CDDP is taken up by the cells is not fully understood [31]. Because the rate-limiting factor of CDDP uptake is its extracellular concentration, uptake is not inhibited by structural analogues, and uptake is not saturable, it has been suggested that CDDP enters the cells by passive diffusion [32]. There is, however, some evidence that indicates that CDDP uptake is mediated by membrane proteins. For example, a variety of pharmacological agents that do not alter the permeability of the membrane inhibit CDDP uptake. Thus, the sodium-potassium ATPase inhibitor ouabain inhibits drug uptake, and CDDP accumulation is potassium-dependent, even though CDDP is not transported through the sodium-potassium pump, suggesting that accumulation is dependent on cell membrane potential [33]. Moreover, several aldehydes inhibit uptake, presumably by forming Schiff bases with membrane proteins [32]. These results suggest a carrier-mediated transporter system for the cellular uptake of CDDP. Interestingly, a 48 kDa membrane protein is expressed in lower levels in CDDP-resistant cells that show decreased CDDP content, indicating that this protein might be involved in CDDP uptake [34]. It is noteworthy that the maximum inhibition of CDDP uptake afforded by the aforementioned compounds is 50%. Accordingly, Gamely and Howell propose that approximately one-half of drug uptake takes place by passive diffusion and the other half occurs by facilitated diffusion through an as yet unidentified pump [35].

In addition to the mechanism described above, platinum in the cells is actively effluxed in an ATP-dependent manner, which causes a decreased intracellular concentration of platinum. The efflux pump for platinum has not yet been defined, although Ishikawa and Oli-Osman [36] have proposed that a glutathione-S-X (GS-X) pump actively effluxes the glutathione-S-platinum (GS-Pt) complex. Experiments using membrane vesicles of tumor cells demonstrate that the

GS-X pump carries GS-Pt complex in an ATP-dependent manner. Leukotriene C₄, a glutathione-S conjugate, is a good substrate for the GS-X pump. The activity of the GS-X pump is competitively inhibited by the GS-Pt complex and ONO-1078 (ONO Pharmaceutical, Osaka, Japan) a leukotriene C₄/D₄ receptor antagonist [37]. These findings suggest that the GS-X pump is the CDDP-efflux pump and that inhibitors of the GS-X pump can reverse CDDP-resistance by inhibiting the activity of the efflux pump.

The second mechanism of CDDP resistance involves many proteins and enzymes such as metallothioneins (MT) and glutathione (GSH)-related metabolism enzymes. MT are stress-response proteins that protect cells and are involved in the detoxification of heavy metals and confer anticancer drug resistance in tumor cells *in vitro* [38]. The expression of MT is often increased in various CDDP-resistant tumor cells [39] and MT gene-transfected tumors are resistant to CDDP, melphalan, chlorambucil and low levels of doxorubicin [40]. In an immunohistochemical study, Matsumoto *et al.* showed a significantly higher proportion of tumors positive for MT among those patients who had received a preoperative course of chemotherapy containing CDDP than in tumors from previously untreated patients [41]. These results suggest that MT are induced by chemotherapy and that such expression may lead to acquired anticancer drug resistance. Inactivation of CDDP by conjugation with GSH [42], which is catalyzed by glutathione transferase (GST) and prevention of CDDP-adducts which is mediated by GSH [43] are also likely to be other CDDP-resistance mechanisms. GSH is essential for the synthesis of DNA precursors, deoxyribonucleotide triphosphates, and indirectly influences DNA repair [44]. Glutathione peroxidase (GPX) catalyzes the oxidation of reduced GSH and, in this process, acts as an effective scavenger of cytotoxic oxyradicals or lipid peroxides that are induced by chemotherapy. Even if the major mechanism of CDDP action is the generation of crosslinks in DNA, CDDP-induced lipid peroxidation has also been reported [45]. GST catalyzes the conjugation of electrophilic metabolites to GSH which leads to their excretion. Recent studies have indicated that GST- may play an important role in the resistance of cancer cells to alkylating agents, including platinum compounds [46]. Furthermore, GST- has been reported as the predominant isoenzyme among three classes of GST in NSCLC tumor tissues [47] and an *in vitro* study has suggested that cellular GST- levels in lung cancer cell lines may reflect some intrinsic resistance to CDDP [48]. GSH reductase, as opposed to GPX, reduces oxidized GSH. Under normal steady-state conditions, most GSH exists in the reduced form. The maintenance of high intracellular concentrations of reduced GSH, requires stringent regulation of GPX activities.

Another mechanism contributing to CDDP-resistance is altered capacity DNA repair. O⁶-alkylguanine-DNA alkyltransferase (ATase) is considered to be a major factor of resistance to alkylating agents. ATase transfers alkyl groups from O⁶-alkylguanine to an internal cysteine in a stoichiometric reaction without regeneration of the alkyl acceptor site. Alterations of ATase expression have been reported in cells resistant to important anti-neoplastic drugs including chloroethylating and methylating agents [49]. Removal of adducts from genomic DNA is mediated by nucleotide

excision repair (NER). NER can be subdivided into genetically separable pathways termed transcription-coupled repair (TCR) and global genomic repair (GGR). TCR repairs transcription blocking lesions in transcribed DNA strands of active genes, whereas GGR repairs the lesions in the non-transcribed strand of active genes and non-transcribing genomes [50, 51, 52]. In the TCR pathway, RNA polymerase II is the sensor for CDDP-induced damage [52]. When transcribing RNA polymerase II encounters the lesion, two transcription-coupled repair-specific factors, CSA and CSB, are involved in the activation of the common NER molecular pathway [53, 54]. The clinical implications of TCR lie in the fact that CDDP-resistant tumors show an intact TCR system, while tumors are sensitive to CDDP when the TCR subpathway is deficient. For GGR, the XPC complex is activated. Along with the basal transcription factor (TFIIH), an XPG binds to the DNA lesion. TFIIH contains two helicases, XPB and XPD, which open an approximately 30-base-long DNA segment around the lesion. The DNA strand that contains the damaged base(s) is excised by the two NER endonucleases, XPG and XPF/excision repair cross complementing 1 (ERCC1). XPG cleaves the damaged DNA strand 3' from the lesion, and XPF/ERCC1 cleaves the damaged strand 5' from the DNA lesion. Finally, the resulting gap is filled in by DNA polymerases in presence of replication factors [54]. Further studies showed that mismatch repair is involved in resistance to CDDP. Thus, CDDP-resistant cell lines after exposure to the drug *in vitro* frequently acquire the mutator phenotype associated with mismatch repair defects and usually contain mutated *hMLH1* genes [32]. Complementary studies with mismatch repair deficient cells have shown that inactivation of mismatch repair genes confers resistance to CDDP. However, the only clinical study which evaluated the importance of mismatch repair found no association between clinical response to CDDP, overall survival and the expression of *hMSH2* and *hMLH1* in paired tumors of ovarian cancer patients [55].

Homologous recombination (HR) represents another DNA repair mechanism involved in resistance to platinum. The importance of recombination in the survival of CDDP-treated cells was first observed by Beck and Brubaker [56], who demonstrated that *recA*, *recB*, and *recC* expressing cells are more sensitive to CDDP than wild type cells, and cells coexpressing *recB recC* are more sensitive to CDDP than either mutant alone. Further studies showed that HR repaired interstrand cross links [57]. However, the repair of interstrand cross links most likely also requires the other pathway, such as NER [32]. Recently, Crul *et al* demonstrated that HR was involved in the synergistic interaction between CDDP and gemcitabine in CHO cell lines. This drug combination has shown efficacy in a number of malignancies including NSCLC, head and neck, urothelial, cervical cancer and synergistic cytotoxicity in a number of cell lines [58]. The authors observed a loss of synergy of both drugs in HR-deficient cell lines suggesting a role for this DNA repair mechanism. The same team has previously investigated the interaction between CDDP and irinotecan in lower and higher eukaryotes and found that DNA repair, in particular homologous recombination, was required for the synergism occurring with these agents [59]. Importantly, CDDP/gemcitabine synergism has been shown

to involve the NER pathway. Expression of ERCC1 antisense RNA abrogates gemcitabine-mediated cytotoxic synergism with CDDP *in vitro* in human colon cancer cells defective in mismatch repair but proficient in NER [60].

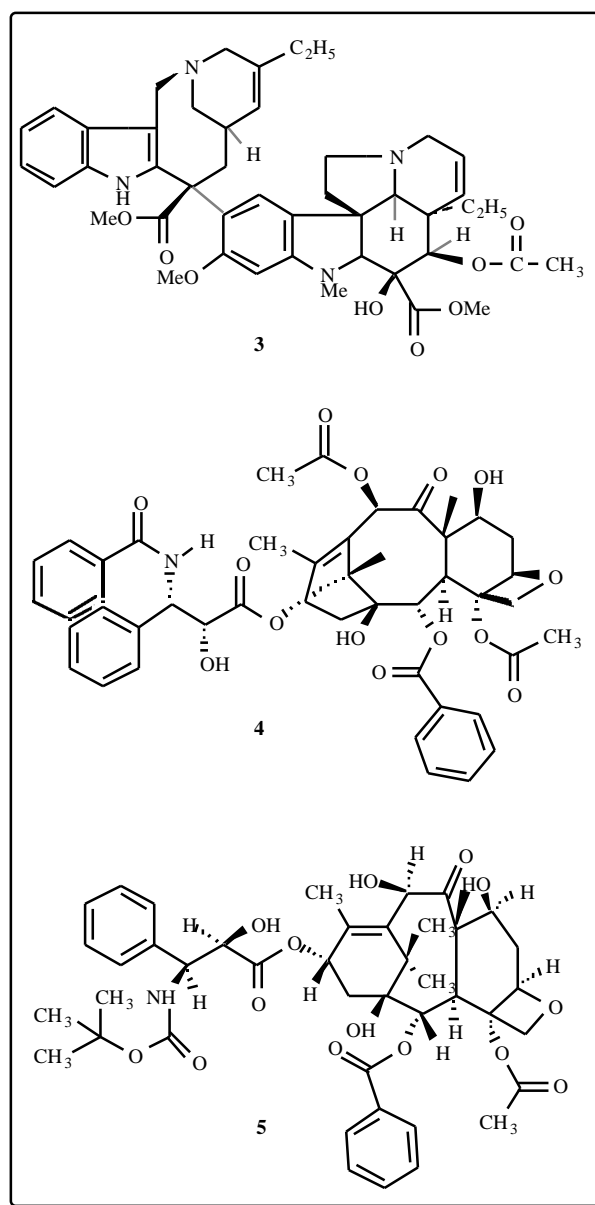
VINOURELBINE AND TAXANES

Vinorelbine and taxanes (paclitaxel and docetaxel) are part of the antitubulin agents whose mechanism of action and resistance are similar [61]. Microtubules are long, filamentous, tube-shaped polymers that are essential in all eukaryotic cells. They are crucial in the development and maintenance of cell shape, in the transport of vesicles, mitochondria and other components throughout cells, in cell signalling, and in cell division and mitosis. Microtubules are composed of polymers of tubulin and microtubules associated proteins (MAPs) in dynamic equilibrium with tubulin heterodimers composed of α and β protein subunits. At least, six α - and seven β -tubulin isotypes have been described with a complex pattern of distribution in various tissues [62]. There are many different MAPs such as MAP2, MAP4, MIP-90, tau, and STOP as well as many microtubule-regulatory proteins, such as survivin, stathmin, TOG, MCAK, MAP4, EB1, dynactin 1, RAC1 and FHIT [62].

The polymerisation of microtubules occurs by a nucleation-elongation mechanism in which the relatively slow formation of a short microtubule nucleus' is followed by rapid elongation of the microtubule at its ends by the reversible, non-covalent addition of tubulin dimers. As a result of this mode of microtubule polymerization, there is an inherent heterogeneity between the two ends of the microtubule, resulting in different kinetics of the addition and subtraction of heterodimers [63]. At the so-called 'plus' end, the kinetics of the polymerisation and depolymerisation are faster than those at the other end, the slower so-called 'minus' end. Recent studies showed that two types of dynamic behaviour determine microtubule structure. The first one is 'dynamic instability' (the fluctuations between phases of elongation and shortening) which is the process by which microtubules polymers undergo prolonged periods of assembly followed by rapid periods of disassembly [64]. Depending on the amount of free tubulin and the tubulin isotype composition, various microtubule-interacting proteins might alter the frequency of the catastrophe and rescue (i.e. rapid depolymerization and polymerization) of the polymer growth and shortening. The other intrinsic property of microtubules is 'treadmilling', originally described *in vitro* by Margolis and Wilson and *in vivo* by Mitchison [65]. Treadmilling is a process by which microtubules incorporate microtubules dimers at their plus end and release them from the minus end. By coupling GTP hydrolysis to the growth and shortening of the microtubule, the length of the polymer remains constant while there is a continuous flux of tubulin dimers at the microtubule plus end and minus end.

Antitubulin agents are believed to block cell division by interfering with the function of the mitotic spindle, blocking the cells at the metaphase/anaphase junction of mitosis. *Vinca* alkaloids, such as vinorelbine "compound (3)" were the earliest tubulin-agents to be used as antimetotics and have

been described as "microtubule depolymerising agents". At high concentrations, these agents reduce or abolish the microtubule content of cells in culture and prevent polymerization of purified tubulin *in vitro*. Conversely, the taxanes (paclitaxel "compound (4)" and docetaxel "compound (5)") promote the polymerization of purified tubulin *in vitro*, at high concentration, and, enhance the fraction of polymerized tubulin in cells. They have been referred to as "microtubule stabilizing agents". This old classification of drugs as microtubule "stabilizers" or "destabilizers" is overly simplistic and can lead to confusion. The reason is that drugs that increase or decrease microtubule polymerization at high concentrations powerfully suppress microtubule dynamics at 10-100-fold lower concentrations and, therefore, kinetically stabilize the microtubules, without changing the microtubule- polymer mass [62]. In other words, the effect of the drugs on dynamics are often more powerful than their effects on polymer mass. It now



seems that the most important action of these drugs is the suppression of spindle-microtubule dynamics by inhibition of spindle treadmilling and dynamic instability, which results in the slowing or blocking of metaphase-anaphase transition and induction of apoptotic cell death [66].

Tubulin and microtubules are the main targets of the *Vinca* alkaloids [67]. Vinorelbine binds to the β -subunit of tubulin dimers at a distinct region called the *Vinca*-binding domain. Vinblastine, the prototype of the *vinca* alkaloids binds both to soluble tubulin and directly to microtubules. As previously described, these drugs depolymerize microtubules and destroy mitotic spindles at high concentrations (for example, 10-100 nM in HeLa cells), therefore leaving the dividing cancer cells blocked in mitosis with condensed non segregated chromosomes) [68]. At low concentrations, vinblastine does not polymerize spindle microtubules, yet it powerfully blocks mitosis and cells die by apoptosis. Studies from Mary Ann Jordan's laboratory indicate that the block is due to the suppression of microtubule dynamics rather than microtubule depolymerization [69]. Thus, the binding of one or two molecules per microtubule plus end is sufficient to reduce both treadmilling and dynamic instability by ~ 50%, without causing appreciable microtubule depolymerization.

Paclitaxel and its semi-synthetic analogue docetaxel have been discovered more recently. The electron crystallographic structure of tubulin has revealed that paclitaxel binds β -tubulin within the lumen of the microtubule [70, 71]. This binding event affects a protein loop, called the M-loop, which is thought to stabilize lateral interactions between the adjacent protofilaments of microtubules. However, other studies using fluorescent analogues of paclitaxel have found conflicting evidence for both the accessibility of the site and the orientation of the drug within the site [72]. Docetaxel is a second generation taxane derived from the needles of the European yew tree which has a wide spectrum of antitumor activity. It exhibited a greater cytotoxicity relative to paclitaxel in several tumor cell lines and linear pharmacokinetics and, due to differences in drug efflux is retained intracellularly for a longer period [73]. Although docetaxel and paclitaxel share a mutual binding site (for which docetaxel has a higher affinity [74]), there is evidence that they have distinct effects on microtubule dynamics [75]. This may underlie the greater potency of docetaxel as a tubulin assembly promoter and microtubule stabilizer compared to that of paclitaxel [76]. In contrast with the large numbers of taxane molecules that are required to increase microtubule polymerization, Mary-Ann Jordan's team found that the binding of a very small number of paclitaxel molecules powerfully stabilizes the dynamics of the microtubules without increased microtubules polymerization. For example, a single paclitaxel molecule bound per several hundred tubulin microtubules can reduce the rate of extent of microtubule shortening by ~ 50% [77]. As with the *vinca* alkaloids, the suppression of spindle-microtubule dynamics prevents the dividing cancer cells from progressing from metaphase into anaphase and the cells eventually die by apoptosis [78].

Several fundamental studies on human cancer lines showed synergy between *Vinca* alkaloids and taxanes [79, 80]. However, Monnier *et al.* who studied the effects of the combination of docetaxel and vinorelbine in 26 chemo-

therapy-naïve patients with NSCLC, reported substantial hematologic and mucosal toxicity, with two toxic deaths [81], and studies of paclitaxel-vinorelbine combinations showed severe and/or frequent neurotoxicity [82, 83].

The best described mechanism of resistance to tubulin-binding agents is the MDR phenotype which has been detailed elsewhere. Both the *vinca* alkaloids and the taxanes are good substrates for the 170 efflux pump [84, 85]. In a number of cases, development of cell lines resistant to vincristine or paclitaxel has been shown to be associated with the expression of *mdr1* [86]. The MRP has also been shown to be an efficient transporter of *vinca* alkaloids, but not taxanes [87].

Altered metabolism and/or subcellular distribution, alterations of the interaction between drugs and their target (microtubules), and altered response to cell cycle arrest induced by mitotic blockage are among the possible non-MDR mechanisms of resistance to tubulin-binding agents (Fig. (1)).

The available data suggest that alterations in microtubule structure and/or function represent an important, and potentially complex, mechanism of resistance to tubulin-binding agents. A number of cell lines resistant to tubulin-binding agents *in vitro* have been shown to contain tubulin alterations in terms of total tubulin content, tubulin polymerization, or tubulin isotype content [88-90]. Jaffrezou *et al.* reported that the KPTA5 cell line, which is exclusively resistant to taxanes, displays increased expression of the class IVa tubulin isotype [90]. Conversely, the KCVB2 cell line, which does not express *mdr1*, is cross resistant to *vinca* alkaloids and to taxanes and has a reduced amount of total tubulin, a higher percentage of polymerized tubulin, and a higher content of class III tubulin isotype [91]. Various investigators have reported altered expression of tubulin isotypes in resistant cell lines [89, 92]. Haber *et al.* reported that in the murine cell line J774, resistance to paclitaxel is associated with a 21-fold increase in class II beta-tubulin isotype [93]. Mutations might also develop in the genes encoding α - and β -tubulin subunits and thus prevent binding of drugs [94, 95].

Another mechanism of resistance to antitubulin agents is altered programmed cell death (apoptosis). The mechanism by which mitotic blockage induces apoptosis remains to be determined, although it is increasingly clear that a number of regulatory molecules [96, 97] as well as oncogenes [98], bind to the mitotic apparatus (Bim, FHIT, cyclin B, c-MYC...). It is highly probable, although the mechanism is poorly understood, that genes that protect cells against apoptosis, such as mutant p53, bcl-2, and bcl-x, may induce resistance to tubulin-binding agents [99, 100]. MAPs are also likely to be involved in mechanisms of resistance to drug-induced apoptosis. MAP4, the expression of which is negatively regulated by wild-type p53 has been shown to increase sensitivity to paclitaxel [101, 102]. Tau overexpression has been described in estramustine-resistant human prostatic carcinoma cells [103].

The relationship between p53 alterations and sensitivity to antitubulin agents is complex. Functional p53 causes cell cycle arrest in the G1 phase in case of DNA damage, thereby allowing DNA repair and enhanced survival in normal cells.

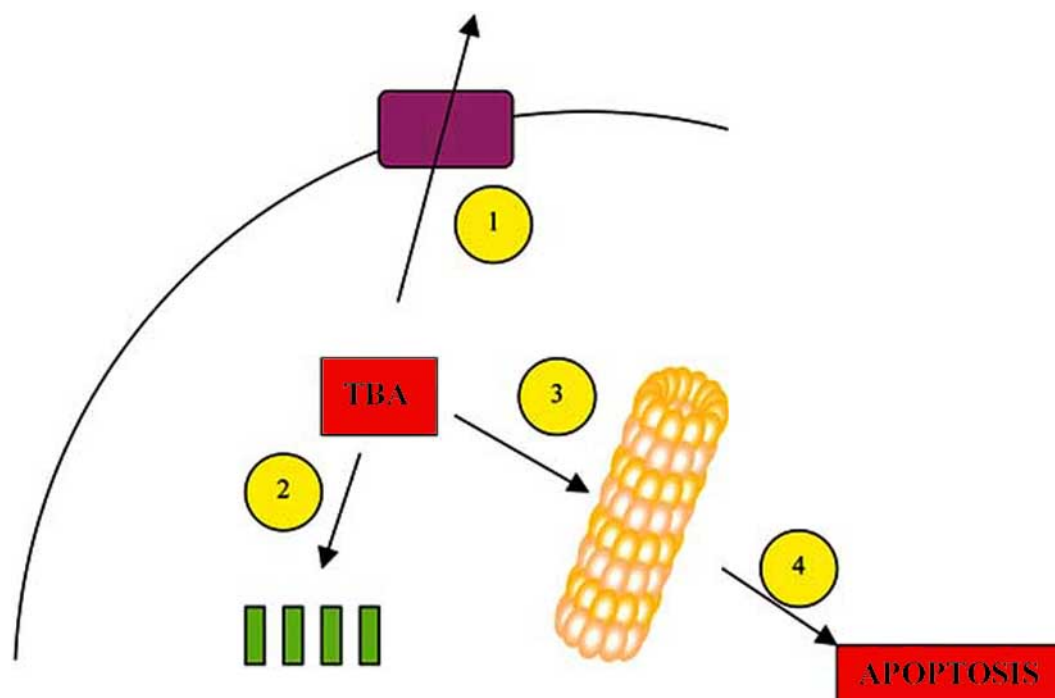


Fig. (1). Potential mechanisms of resistance to tubulin-binding agents (TBA) 1: Efflux of drug by a membrane pump. 2: Altered metabolism or distribution of agent. 3: Altered interaction of agent with microtubules. 4: Inadequate induction of apoptotic signal.

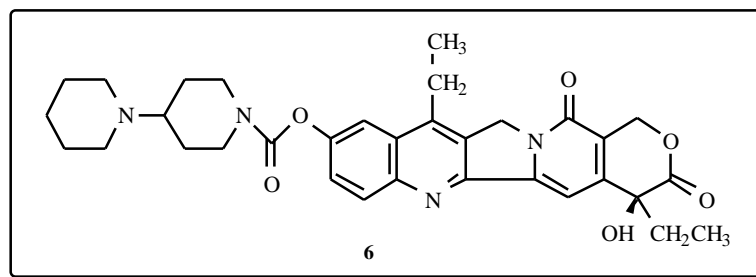
It was thus expected that abnormal p53 would sensitize tumor cells to DNA damaging agents. In most cases, however, abnormal p53 was associated with drug resistance. These unexpected findings were attributed to the fact that tumor cells that did not express functional p53 were unable to initiate apoptosis because of the DNA damage they had sustained. The temporary inactivation by acute human papillomavirus or the permanent inactivation obtained in mice is associated with increased sensitivity to paclitaxel [104, 105]. Woods *et al.* suggested that paclitaxel induces apoptosis through two different pathways: a p53-independent pathway occurred in cells blocked in prophase, which is observed in p53-expressing and in p53-null mouse embryo fibroblasts; and a p53-dependent mechanism, which occurs in cells that accumulate in G1 and requires functional p53 [106]. The observation by various authors that *vinca* alkaloids and paclitaxel induce p53 may thus be interpreted as a resistance mechanism of the cells against the cytotoxic effect of paclitaxel [107]. Paclitaxel has been shown to modulate the level of expression of genes involved in apoptotic regulation, such as bcl-x_L [108]. Antimicrotubules are believed to cause inactivation of Bcl-2 function through

phosphorylation [109]. Docetaxel is 10- to 100-fold more potent than paclitaxel in phosphorylating Bcl-2 and this may account for the differential pro-apoptotic activity of docetaxel compared with paclitaxel.

IRINOTECAN

Irinotecan is a semisynthetic analog of camptothecin characterized by the presence of a bulky piperidino side-chain at the C-position “compound (6)” which causes S-phase specific cell killing by poisoning Topo I in the cell [110]. Irinotecan has been approved for use in combination with 5-FU/leucovorin as a first line treatment for metastatic colorectal cancer [111] and shown exciting results from a randomized trial in combination with CDDP in patients with extensive small-cell lung cancer [112]. In NSCLC, the combination of CDDP and irinotecan is at least comparable with other active combination regimens.

Understanding the pharmacology of irinotecan provides insights into the basis of irinotecan tumor resistance [110]. The side-chain is cleaved enzymatically by carboxylesterase to 7 ethyl-10-hydroxycamptothecin (SN-38), which is 1,000-



fold more potent than irinotecan. Both irinotecan and SN-38 are in equilibrium with their active lactone and inactive carboxylate forms. Genetic variability of carboxylesterase expression and/or activity is suspected, as variations of SN-38 levels are observed among different individuals for a given dose of irinotecan [113]. After conversion from irinotecan by carboxylesterase, SN-38 is deactivated through conjugation by uridine diphosphate glucuronosyltransferase isoform 1A1 (UGT1A1) in the liver to an inactive form. Patients affected by Gilbert's syndrome or Crigler-Najjar syndrome type I in which UGT1A1 is respectively mutated or deficient are at increased risk for severe irinotecan-induced toxicity. The elimination of irinotecan and SN-38 is through biliary excretion, which is dependent on the canalicular multispecific organic anion transporter, a member of the transporters with an ABC [114]. Subsequently, SN-98 is deconjugated to SN-38 by β -glucuronidase produced by the intestinal bacterial flora, which may account for the late SN-38 double peaks in the plasma, and may be responsible for the delayed intestinal toxicity of irinotecan [115]. Thus, enzyme activity variations due to genetic polymorphism or drugs interaction may contribute to plasma concentration variations and to drug resistance.

Irinotecan interacts with cellular Topo I-DNA complexes. This complex is not lethal to the cells by itself; however, upon their collisions with the advancing replication forks, the formation of a double-strand DNA occurs, leading to irreversible arrest of the replication fork [116], G2 arrest/delay by signalling the presence of DNA damage to an S-phase checkpoint mechanism [117] and cell death. Non S-phase cells can also be killed and the mechanism appears to be related to transcriptionally mediated DNA damage and through the mechanism of apoptosis [118].

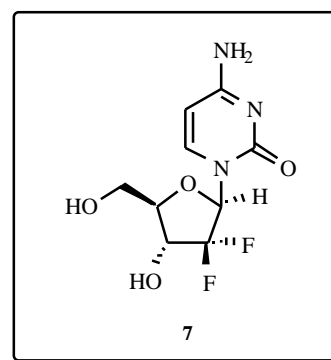
At the tumor cell level, mechanisms of irinotecan resistance involve: (1) decreased intracellular levels of irinotecan by active efflux; (2) Decreased Topo I levels; (3) Topo I mutations; (4) altered DNA repair and (5) Nuclear factor kappa B activation (NF- κ B).

As described previously, P-gp, MRP family of transporters as well as BRCP play important roles in the efflux and active excretion of irinotecan. Experimental studies from yeast systems and mammalian cell lines showed that irinotecan cytotoxic effects are proportional to cellular levels of Topo I [119, 120]. Although no consistent association has been described between pre-treatment tumoral Topo I expression and antitumor response to irinotecan, these questions has not been properly addressed in prospectively designed clinical trials. Either increased transcription or increased mRNA stability may contribute to irinotecan resistance. On the other hand, specimens with reduced expression were found to have a non productive rearrangement of the Topo I genome, leading to decreased transcription and thus reduced enzymatic expression of topo I [121]. Fundamental studies in certain cell lines demonstrated that resistance to camptothecin was related to mutations in Topo I [110, 122]. Mutations have been found in the area that may alter DNA cleavage or Topo I-DNA-irinotecan interactions, so that camptothecin can no longer enter the complex [123], or in the region immediately flanking the catalytic tyrosine that may affect the activity of the enzyme [124]. Another

cellular mechanism of resistance to irinotecan is repair of irinotecan-induced damage; a mechanism which is coupled with RNA transcription [110]. The collision between the elongation RNA polymerase complex and the Topo-I cleavable complex triggers degradation of Topo I through an ubiquitin/26 S proteasome system [125]. Subsequently to Topo I destruction, repair of the single-strand break can presumably occur. Fundamental studies showed that the most sensitive cell lines were completely defective in irinotecan-induced Topo I reduction. Another mechanism of resistance involves activation of the transcription factor nuclear factor Kappa B and subsequent suppression of apoptosis. NF- κ B suppresses the apoptotic cascade induced by tumor necrosis factor-alpha (TNF- α), oncogenic Ras, and chemotherapy agents, particularly irinotecan. Recent studies revealed that activation of NF- κ B increased two inhibitors proteins involved in the c-Jun N terminal kinase (JNK) pathway which then inhibits JNK signalling by TNF- α [126, 127].

GEMCITABINE

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is a deoxycytidine analogue with two fluorine substituted for two hydrogens atoms in the 2' position of the deoxyribose sugar ("compound (7)"). Unlike others nucleoside analogues which are an important class of antimetabolites used in the treatment of hematological malignancies, gemcitabine has activity against pancreatic, lung, breast and bladder cancers [128]. Extensive search has been performed to elucidate the complex mechanism of action of this relatively new drug [129] (Fig. (2)). Like other nucleoside analogs gemcitabine is hydrophilic and required specialized transport systems.



Three out of the seven distinct carriers for the transport of nucleosides are involved in gemcitabine transport: the two sodium-independent (equilibrative) transporters type (hENT1 and hENT2) and one sodium-dependent (concentrative) type (hCNT1) [130]. Once inside the cell, dFdC is first phosphorylated into dFd-CMP by deoxycytidine kinase (DCK) [131]. It is subsequently phosphorylated by pyrimidine kinases to the active 5'-diphosphate (dFd-CDP) and triphosphate (dFd-CTP) derivatives [132]. The mitochondrial enzyme thymidine kinase 2 phosphorylates the natural nucleosides thymidine and deoxycytidine, although, to a lesser extent than DCK [133]. Inactivation of gemcitabine can occur by deamination into its inactive metabolite 2',2'-

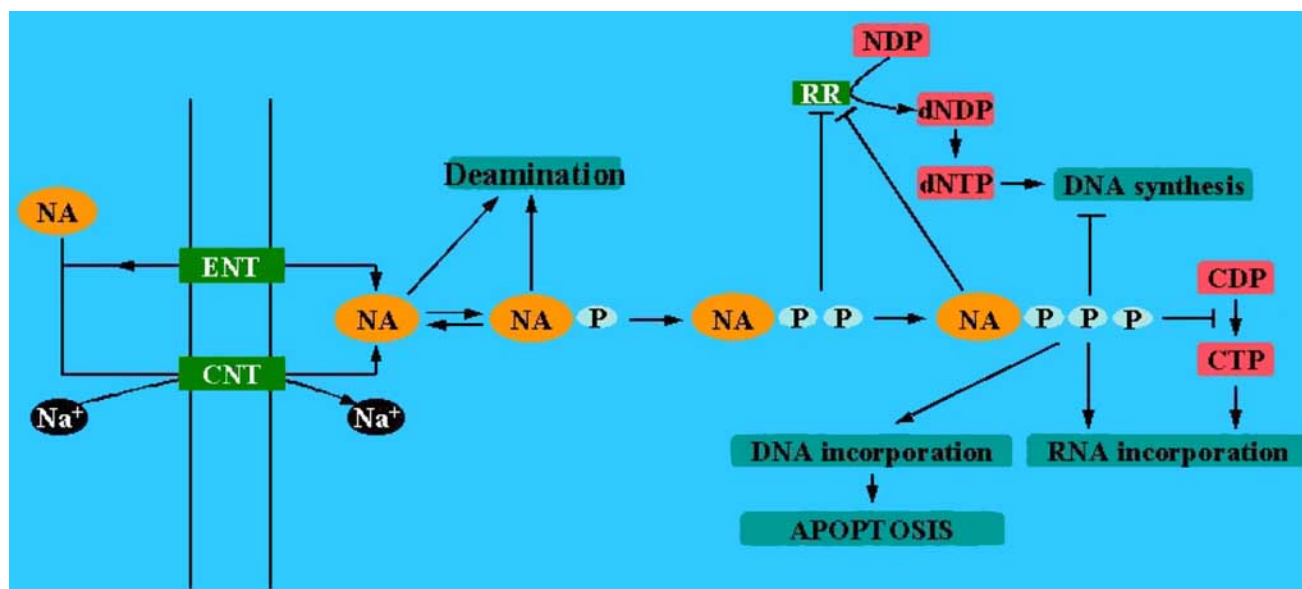


Fig. (2). Metabolism and activity of gemcitabine. G, gemcitabine; hENT, human Equilibrative Nucleotide Transporter; hCNT, human Concentrative Nucleotide Transporter; RR, Ribonucleotide Reductase.

difluoro-2'-deoxyuridine (dFd-U) by cytidine-deaminase (CDD) [134] or dephosphorylation of dFd-CMP by 5' nucleotidase (5'NT) [135]. Another pathway of inactivation is deamination of dFd-CMP to 2',2'-difluoro-2'-deoxyuracil monophosphate (dFd-UMP) by the action of deoxycytidilate deaminase. dFd-CTP is incorporated into DNA by replication synthesis in the C sites of the growing DNA strand [136]. Once incorporated into the DNA strand, an additional natural nucleoside is added masking gemcitabine and preventing DNA repair by base pair excision. Thereafter, the DNA polymerases are unable to proceed [137], a process designated as masked DNA chain termination. The active diphosphate metabolite of gemcitabine also inhibits DNA synthesis indirectly through inhibition of ribonucleotide reductase (RR). RR converts ribonucleotide 5'-diphosphates to deoxyribonucleotide 5'-diphosphates, which are essential for DNA synthesis. This effect blocks *de novo* DNA synthesis pathway and self-potentiates gemcitabine activity by decreasing intracellular concentrations of normal deoxynucleotide triphosphates (dNTPs) (particularly dCTP). Reduction in cellular dCTP results in increased gemcitabine nucleotide incorporation into DNA and increased formation of active gemcitabine di and triphosphates, since DCK is down-regulated by high cellular dCTPs levels. Possible inhibition of thymidilate synthase by the deaminated product dFdUMP might affect DNA synthesis resulting in DNA damage [138]. Other self-potentiating mechanisms of gemcitabine include a decreased elimination of gemcitabine nucleotides by direct inhibition of CDD [139]. Gemcitabine is not only incorporated into DNA, but also into RNA, subsequently inhibiting RNA synthesis [140].

The mechanism by which gemcitabine induces cell death is primarily by apoptosis, although the exact molecular events responsible for triggering are not known [129].

The spectrum of activity of gemcitabine in solid tumors is related to specific characteristics. Compared with other nucleoside analogues, gemcitabine serves as better transporter substrate for membrane pumps, is phosphorylated more efficiently, and is eliminated more slowly, favoring a longer retention time of the active form in tumor cells [139]. This prolonged presence of active gemcitabine derivatives may explain part of its effect on slowly dividing tumor cells. Gemcitabine is frequently combined with CDDP (4), and the extent of platinum-DNA adduct formation positively correlates with the level of incorporation of dFdC into DNA [140].

Three general mechanisms of resistance have been described in cell lines and in clinical samples. A primary mechanism of resistance arises from an insufficient intracellular concentration of gemcitabine triphosphates because of a defective activation/degradation pathway. This may result from inefficient cellular uptake, reduced levels of the activating enzyme DCK and increased gemcitabine degradation by increased CDD or 5'NT activity. Indeed, Mackey *et al.* found that cells with a nucleoside-transporter deficiency were highly resistant to gemcitabine [141]. Numerous studies showed a relationship between DCK activity and sensitivity of solid tumors and hematological malignancies to gemcitabine [129, 142]. Interestingly, Ruiz van Haperen *et al.* described a cell line with reduction in the thymidine kinase 2 enzyme as possible explanation for its resistance to gemcitabine [140]. We have demonstrated that the resistance of the gemcitabine-variants of the erythroleukemic cell K562 was associated with an increased 5'NT activity [143]. Neff and Blau showed that human hematopoietic CEM cells transfected with CDD were 2.5 fold resistant to gemcitabine [144]. The second type of resistance mechanism concerns altered interactions with

intracellular targets such as RR becoming insensitive to inhibition by gemcitabine. Goan *et al.* demonstrated a 2-fold increase RR activity in the human oropharyngeal epidermoid carcinoma KB cells resistant to gemcitabine, resulting in increased dCTP pools [145]. Finally, alterations of apoptosis-regulating genes such as p53 also appear to be involved in the sensitivity of tumor cells to the cytotoxic effect of gemcitabine [146]. We have shown that inactivation of p53 in the mammary adenocarcinoma line MCF-7 confers resistance to gemcitabine toxicity [147]. The resistance phenotype was related to a higher degree apoptosis in MN-1 cell line containing the wildtype p53 (wt-p53) than in the MDD2 line containing a dominant negative variant of the p53 protein (mut-p53). This corresponded with suppression of Bcl-2 and Bcl-X/L suppression in wt-p53 cells exposed to gemcitabine whereas Bcl-2 levels remained stable and Bcl-X/L levels increased in mut-p53 cells exposed to gemcitabine. A more pronounced block in G1 phase was observed in MN-1 cells. We concluded that loss of p53 function lead to loss of cell cycle control and alterations in the apoptotic cascade, conferring resistance to gemcitabine in cancer cell lines displaying a mut-p53.

CLINICAL STUDIES

Several clinical studies recently showed DNA and RNA levels may predict prognosis and the response to specific cytotoxic drugs in treated NSCLC patients.

Monzo *et al.* showed that, in lung tumors, mutations of class I β -tubulin, which represents the most abundant tubulin isotype, were correlated with adverse outcomes in term of response and survival. Among 49 NSCLC patients who had received paclitaxel, 16 patients (33%) had class I β -tubulin mutations, including major sequences alterations (frame-shifts or premature codons) in 6 cases [148]. None of the patients with β -tubulin mutations had an objective response whereas 13 of 33 patients (39.4%) without β -tubulin mutations had complete or partial response. Nevertheless, conflicting data were published elsewhere [149, 150, 151]. Sale *et al.* failed to identify any mutations in DNA isolated from 17 paclitaxel-naïve specimens of NSCLC with denaturing high pressure liquid chromatography and direct sequence analysis [149]. In another study, Tsurutani *et al.* investigated the frequency of β -tubulin mutations in Japanese patients with NSCLC, by direct sequence analysis following reverse transcription-polymerase chain reaction (RT-PCR) of the β -tubulin gene [150]. First strand cDNA sequence analysis 42 samples showed silent mutations at codon 180 of the β -tubulin gene, which encodes the GTP-binding site of the protein, and codons 195 and 217. However, neither missense nor non-sense mutations affecting microtubule dynamics, within or near the GTP-binding site of the β -tubulin gene, were detected. In the Kelley *et al.* study only 2/25 NSCLC cell lines were described as having either silent mutations or polymorphisms [151]. These results indicate that the Monzo study may have overestimated the incidence of β -tubulin mutations as a result of the existence of pseudogenes, since genomic DNAs only were used to analyze the β -tubulin gene in tumor specimen.

Rosell *et al.* used RT-PCR to analyze expression of β -tubulin III, stathmin, RRM1, UGTP1 and GSTP1 in mRNA

isolated from paraffin-embedded tumor biopsies of 75 NSCLC patients treated as part of a large randomized trial [152]. Twenty-two patients were treated with gemcitabine/CDDP, 25 with vinorelbine/CDDP, and 28 with paclitaxel/carboplatin. Patients with low β -tubulin III levels had better response in the paclitaxel/carboplatin arm ($P=0.05$), and those with low RRM1 levels showed a tendency to better response in the gemcitabine/CDDP arm. Time to progression was influenced by β -tubulin III ($P=0.03$) and stathmin ($P=0.05$) levels in the vinorelbine/CDDP arm and there was a tendency toward correlation between β -tubulin III and time to progression in the paclitaxel/carboplatin arm. RRM1 levels influenced time to progression ($P=0.05$) and even more so, survival ($P=0.0028$) in the gemcitabine/CDDP arm. In a recent study, Rosell *et al.* examined the potential correlation and predictive value RRM1 in 67 resected specimens from 67 stage IIB, IIIA, and IIIB NSCLC patients treated with neoadjuvant gemcitabine/CDDP followed by surgery [153]. Applying RT-PCR to paraffin-embedded surgical specimens, they found that patients in the bottom quartile for RRM1 levels had a decreased risk of death compared of those in the top quartile (risk ratio=0.30; $P=0.033$). Median survival for the 17 patients in the bottom quartile was 52 months, whereas for the 15 in the top quartile, it was 26 months ($P=0.018$). Furthermore, the 17 patients in the bottom quartile had better outcomes, including a higher rate of complete resections (94% versus 72%; $P=0.03$), lobectomies (71% versus 34%; $P=0.004$), more radiographic responses (65% versus 54%; $P=0.24$) and complete pathological responses (29% versus 0%; $P=0.0001$). The authors conclude mRNA levels should be additionally validated to proceed with tailored chemotherapy. Nevertheless, these results should be interpreted cautiously, as they concerned treated specimens. Thus, we may expect that the analysed samples concerned selected fragments composed of drug-resistant cells.

In the case of resistance to CDDP, translational research has focused on ERCC1 mRNA overexpression [154]. Lord *et al.* correlated response with ERCC1 expression in 56 patients with advanced NSCLC patients treated with gemcitabine and CDDP. RNA was isolated from paraffin-embedded pretreatment tumor specimens, and relative expression levels of ERCC1/ β -actin were measured by quantitative RT-PCR [155]. There were no differences in ERCC1 levels by gender, age, performance status, weight loss, or tumor stage. Median overall survival was significantly longer in patients with low ERCC1 expression tumors compared to patients with high expression. Although there was a trend toward decreased response with high ERCC1 mRNA levels, this difference failed to reach statistical significance. Also, the same authors recently found that using real-time quantitative RT-PCR assay, ERCC1 and RRM1 correlated with survival in a series of 81 unresectable NSCLC patients [156]. Patients were included in a large randomized trial carried out from September 1998 to July 2000, comparing gemcitabine/CDDP/vinorelbine versus gemcitabine/vinorelbine followed by vinorelbine/ifosfamide. Using the same method, they showed a strong correlation between RRM1 and ERCC1 mRNA expressing levels (Spearman $r = 0.410$; $P<0.001$). Median survival was significantly longer in patients with both low ERCC1 and RRM1 expression tumors compared to

patients with high expression. In the gemcitabine/CDDP arm, patients with low RRM1 mRNA levels had significantly longer median survival times than those with high levels (13.7 versus 3.6 months; 95% confidence interval (CI), 9.6-17.8 months; $P=0.0009$).

The main obstacle to testing drug resistance with these techniques is the scarcity of tumor tissue available for RNA isolation and quantitative PCR. Conversely, XPD can be analyzed in constitutional DNA, for example, in DNA isolated from peripheral blood lymphocytes. Thus, XPD polymorphism has been related to lower DNA repair in the study of Spitz *et al.* [157]. Approximately half of the population examined have the genotype Lys751Lys and also have Asp312Asp. These patients have a good DNA repair capacity and, therefore, are presumably resistant to CDDP. In this epidemiological study matching 341 lung cancer cases with 360 smoker control subjects, a host-cell reactivation assay measuring the activity of the chloramphenicol acetyltransferase gene was used in cells transfected with plasmids treated with benzo[a]pyrene diol epoxide (BPDE). DNA repair capacity was lower in the lung cancer patients than in the controls. The variants Gln751Gln and Asn312Asn had suboptimal DNA repair capacity in contrast with the wild-type genotype both in cases and controls, which exhibited the most proficient DNA repair capacity [157]. In another study, Camps *et al.* examined XPD polymorphisms at codon 751 and 312 in DNA isolated from peripheral blood in 39 patients with gemcitabine/CDDP-treated locally advanced NSCLC [158]. Although no significant correlation was observed between XPD genotype and objective response, a trend toward better response was observed in patients with XPD polymorphism at codon 312. In a recent study, Isla *et al.* assessed whether single nucleotide polymorphisms in ERCC1, XPD, RRM1 and MDR1 predicted survival in 62 docetaxel-CDDP-treated stage IV patients [159]. Patients homozygous for the ERCC1 118 C allele demonstrated a significant better survival and time to progression, whereas no other significant differences were observed. The clinical interest of these findings lies in their potential usefulness in identifying constitutional DNA from lymphocytes, the polymorphisms associated with suboptimal DNA repair capacity and their potential role in identifying patients with better response to CDDP chemotherapy.

NER activity estimated as the DNA repair capacity (DRC) measured in the patients' peripheral lymphocytes may also predict survival in patients with NSCLC. Thus, Bosken *et al.* showed that effective host DRC was associated in multivariate analysis with poorer survival in patients who were treated with chemotherapy [160]. DRC has been assessed in the peripheral blood lymphocytes by the host-cell reactivation assay, which measured cellular reactivation of a reporter gene damaged by exposure to BPDE. Patients were divided into quartiles according to their DNA repair capacity. Patients in the top quartile (DNA repair capacity >9.2%) had a risk of death that was more than two times the risk of death for patients in the bottom quartile (DNA repair capacity <5.8%) ($P=0.01$). Median survival was 8.9 months for patients in the top quartile, compared with 15.8 months for those in the bottom quartile ($P=0.04$). Intriguingly, among the 36 chemo-naïve patients who underwent curative surgical resection, there was a slight survival advantage

associated with increased DRC. This finding could be extremely relevant when interpreting the results of neoadjuvant chemotherapy trials in early NSCLC. The assessment of DNA repair capacity, reflecting the GGR subpathway, is warranted in such trials to identify the subgroup of patients with low DNA repair capacity, which could have lower survival when treated with surgery alone and at the same time could benefit from neoadjuvant or adjuvant chemotherapy. In contrast, patients with high DNA repair capacity could have better survival when treated with surgery alone and could be refractory to neoadjuvant or adjuvant approaches.

UGTP1A1 polymorphisms were analyzed in 46 NSCLC patients treated second-line in with docetaxel and irinotecan and divided according to whether they had 6/6 or 6/7 or 7/7 tandem repeats in the promoter region [161]. Patients with 6/6 repeats had a rate partial response or stable disease of 26% compared with 45% in those were 6/7 or 7/7. The 1-year survival rates in the two groups were 17 and 58%, respectively.

Determination of methylation-induced gene silencing of genes involved in the apoptosis pathway also appeared to be very promising [162]. Aberrant methylation is associated with inappropriate gene silencing which has been implicated in tumor progression through the inactivation of genes thought to be suppressing invasion and metastasis. Conversely, methylation of the death-associated protein kinase (DAPK) gene has been linked to poor survival in stage I NSCLC [163]. DAPK is a novel subfamily of pro-apoptotic serine/threonine kinases involved in both death-receptor and mitochondrial pathway of apoptosis [164]. Hypermethylation of the DAPK gene was found in 59 of 135 NSCLC patients (44%) [163]. The five-year survival rate was 56% for those with hypermethylation and 92% for those without ($P<0.001$).

Immunohistochemical studies may also predict tumor resistance *in vitro* and patient outcome. Volm *et al.* showed a significant relationship between the resistance to doxorubicin measured by *in vitro* short-test and the over-expression of PgP, GST- and the down-regulation of topoisomerase II measured in tissue samples of 94 previously untreated NSCLC [26]. The same author investigated the expression of topoisomerase II, catalase, MT and thymidilate synthase in 48 human squamous cell lung carcinomas and found a significant correlation between resistance to doxorubicin *in vitro* and the over expression of MT and thymidilate synthase, an enzyme involved in 5-fluorouracil metabolism whereas there no relation was found with topoisomerase II and catalase expression [165]. In another study of 94 surgically treated NSCLC, Volm *et al.* demonstrated that protein expression profiles of 40 cellular parameters assessed by cluster analysis was indicative for drug resistance of the tumors to doxorubicin determined using the same short-term *in vitro* test [166]. Three different resistant profiles were defined. In the most frequent drug resistant profile, the resistance proteins PgP/MDR1, thymidilate synthase, GST-, MT, O⁶-methylguanine-DNA-methyltransferase (OMDM) and major vault-protein/LRP were up-regulated. In the second profile, only three resistance proteins were increased (GST-, OMDM and LRP). In the third profile which consisted of few resistant tumors, only five of the resistance

factors were increased (MDR-1, thymidilate-synthetase, GST-, OMDM and LRP). Ogawa *et al.* realised an immunohistochemical study of GPX and glutathione reductase in 70 previously untreated NSCLC and showed an inverse relationship between CDDP sensitivity assessed by *in vitro* DNA histogram and the frequency of enzyme expression [167]. These studies have little clinical relevance. Immunological studies which investigate the relation between the levels of proteins involved in cytotoxic metabolism and response to chemotherapy and patients outcome are of greatest interest. Bai *et al.* showed that negative expression of GST- in tissues from 38 locally advanced or metastatic NSCLC patients was significantly related to response to CDDP based chemotherapy [168]. They were treated with a variety of CDDP-based chemotherapy and there was no significant correlation between GST- expression and the clinicopathologic factors examined (age, sex, performance status, histology, differentiation grade, and stage). Oberli-Schrämli *et al.* analyzed by immunohistochemical methods in parallel PgP expression, GSH content, GST, GPX and ATase activities in tumors of NSCLC and small-cell lung cancer and related them to histopathological and clinical variables and treatment outcome [169]. They found no significant difference in the GSH-system or in the expression of PgP whereas ATase was elevated in tumor tissue of NSCLC only, suggesting a role for this enzyme in inherent drug resistance of NSCLCs. We have shown that high expression of class III tubulin by tumor cells in 19 NSCLC patients receiving a taxane-based regimen was associated with a poor response to chemotherapy and a shorter progression-free survival [170]. These studies suggest that immunohistochemical expression of proteins might be useful for predicting sensitivity to CDDP or taxanes.

Recently, a randomized-study showed that CDDP-based chemotherapy improves survival among patients with completely resected NSCLC [171]. 1867 patients underwent randomization either to three or four cycles of CDDP plus a *vinca* alkaloid (vindesine, vinblastine or vinorelbine) or etoposide. This study demonstrated an absolute five-year benefit in overall survival of 4.1%. Thus, adjuvant chemotherapy represents another area of investigation for pharmacogenomic studies.

CONCLUSIONS

The available knowledge of the predictive value of the target-molecules and the recent gene profiling techniques allow us to expect a new period for chemotherapy in NSCLC. Although NSCLC remains the paramount chemoresistant tumor with dismal prognosis and without apparent differences in survival, regardless of the chemotherapy regimen used, genetic information opens the gates to pharmacogenomic-guided chemotherapy. However, these findings should be tested in prospective customized chemotherapy trials in localized and metastatic disease.

Gene sequencing, mRNA gene profiles, DRC and gene polymorphism determinations but also immunochemistry studies could be used to predict drug resistance. In the near future, microarray technologies should yield quantitative measurements of mRNA from a large number of different

genes simultaneously, thus facilitating the way for molecular tumor profiling and precise tailoring of individual chemotherapy regimens [172]. However, numerous studies have shown some limitations. An essential question is whether the various investigators have simply selected biomarkers of tumor aggressivity rather than of chemoresistance. Indeed, Nooter *et al.* have shown a correlation between *MPRI* and tumor cell differentiation among the squamous cell carcinomas [173]. In the same way, Sugawara *et al.* showed that *MRPI* expression was higher in well-differentiated adenocarcinomas than in poorly differentiated adenocarcinomas (15). In another study, Koomägi *et al.* demonstrated an up-regulation of resistance-related proteins such as glutathione GST-, thymidilate synthase, and PgP in human NSCLCs with poor vascularization investigated by immunostaining of endothelial cells for factor VIII antigen [174]. These data underline the requirement to perform multivariate analyses taking into account classical prognostic factors and the comparison with control groups treated with others regimens.

Chemoresistance to a given compound is complex and multifactorial. Chemoresistance to drug combinations which possess synergistic interactions are even more complex. A better understanding of the mechanisms involved in drug cytotoxicity and drug interactions is therefore required. Recent techniques of gene invalidation, such as RNA interference, are invaluable to dissect pathways involved in drug-induced cell death.

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ABBREVIATIONS

ABC	=	ATP binding cassette
ATase	=	Alkylguanine-DNA alkyltransferase
BPDE	=	Benzo[a]pyrene diol epoxide
BRCP	=	Breast cancer resistance
CDD	=	Cytidine-desaminase
CI	=	Confidence interval
CDDP	=	Cis-platin
DAPK	=	Death-associated protein kinase
DCK	=	Deoxycytidine kinase
dFdC	=	2',2'-difluorodéoxycytidine, gemcitabine
dFd-CDP	=	dFd-C diphosphate
dFd-CMP	=	dFd-C monophosphate
dFd-CTP	=	dFd-C triphosphate
dFd-U	=	2',2'-difluoro-2'-deoxyuridine
dFd-UMP	=	2',2'-difluoro-2'-deoxyuracyl monophosphate
dNTPs	=	Desoxyribonucleotide triphosphates
DRC	=	DNA repair capacity

ERCC1 = Excision repair cross complementing 1
 GGR = Global genomic repair
 GPX = Glutathione peroxidase
 GSH = Glutathione
 GS-Pt = Glutathione-S-platinum
 GST = Glutathione transferase
 GS-X = Glutathione-S-X
 hENT1 = hENT2, human Equilibrative Nucleoside Transporters 1 and 2
 hCNT1 = Human Concentrative Nucleoside Transporters
 ISN-38 = 7 ethyl-10- hydroxycamptothecin
 JNK = c-Jun N terminal kinase
 LRP = Lung resistance-protein
 MAPs = Microtubule associated proteins
 MDR = Multidrug resistance
 MRP = Multidrug resistance protein
 MT = Metallothioneins
 NER = Nucleotide excision repair
 NF- B = Nuclear factor kappa B activation
 NSCLC = Non-small-cell lung cancer
 5'NT = 5'nucléotidase
 OMDM = O⁶methylguanine-DNA-methyltransferase
 PgP = P glycoprotein
 RR = Ribonucleotide reductase
 RRM1 = Ribonucleotide reductase subunit M1
 RT-PCR = Reverse transcription-polymerase chain reaction
 TCR = Transcription-coupled repair
 TNF- = Tumor necrosis factor-alpha
 topo I = Topoisomerase
 UGT1A1 = Uridine diphosphate glucuronosyltransferase isoform 1A1.

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