New insights into the kinetic resistance to anticancer agents

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Abstract

Kinetic resistance plays a major role in the failure of chemotherapy towards many solid tumors. Kinetic resistance to cytotoxic drugs can be reproduced *in vitro* by growing the cells as multicellular spheroids (Multicellular Resistance) or as hyperconfluent cultures (Confluence-Dependent Resistance). Recent findings on the cell cycle regulation have permitted a better understanding why cancer cells which arrest in long quiescent phases are poorly sensitive to cell-cycle specific anticancer drugs. Two cyclin-dependent kinase inhibitors (CDKI) seem particularly involved in the cell cycle arrest at the G1 to S transition checkpoint: the p53-dependent p21^{cip1} protein which is activated by DNA damage and the p27^{kip1} which is a mediator of the contact inhibition signal. Cell quiescence could alter drug-induced apoptosis which is partly dependent on an active progression in the cell cycle and which is facilitated by overexpression of oncogenes such as c-Myc or cyclins. Investigations are yet necessary to determine the influence of the cell cycle on the balance between antagonizing (bcl-2, bcl-X_L...) or stimulating (Bax, Bcl-X_S, Fas...) factors in chemotherapy-induced apoptosis. Quiescent cells could also be protected from toxic agents by an enhanced expression of stress proteins, such as HSP27 which is induced by confluence. New strategies are required to circumvent kinetic resistance of solid tumors: adequate choice of anticancer agents whose activity is not altered by quiescence (radiation, cisplatin), recruitment from G1 to S/G2 phases by cell pretreatment with alkylating drugs or attenuation of CDKI activity by specific inhibitors.

Introduction

Inherent or acquired resistance of tumor cells to available anticancer drugs is the main cause of failure of chemotherapy in common solid tumors such as colon, lung and breast carcinomas. For a long time, resistance of cancer cells to cytotoxic drugs was compared with resistance of bacteria to antibiotics and related to clonal mutations and expansion under the pressure of selection of the inducing agent. This purely genetic theory defended by Goldie and Coldman [1] or Riordan and Ling [2], led to the discovery of the multidrug resistance (MDR) phenotype for anthracyclines and tubulin-inhibitors and of the role of O₆-methylguanine-DNA methyltransferase and glutathione-S-transferase for alkylating agents [3]. However, these genetic models failed to fully explain the resistance observed in patient tumors. The

P-glycoprotein responsible for the MDR phenotype was not identified in some highly resistant tumors such as melanomas. Most clinical trials aiming at circumvent MDR have failed, even in tumors such as kidney and colorectal tumors in which P-glycoprotein was detected at a high level. The genetic theory did not fully explain the tumor mass-related resistance that is daily encountered by oncologists [4]. Infraclinical metastases of osteosarcomas are usually cured by chemotherapy while clinically detectable metastases resist to chemotherapy. A similar situation is observed in breast cancer where adjuvant chemotherapy is effective to avoid relapses after surgery whereas the same protocols delay but do not prevent the fatal evolution of advanced disease.

For a long time, authors explained tumor mass-related resistance by the poor vascularization of large tumors, the low pH [5], and the modifications of the

cell cycle at the inner of these tumors [6,7]. However, the precise mechanisms of the kinetic resistance remained poorly understood. This resistance is clearly an epigenetic phenomenon that affects a broad range of anticancer agents, including cytotoxic drugs and radiations that display their maximal activity at precise phases of the cell cycle. The topoisomerase II-reactive agents such as etoposide and anthracyclines produce double-strand breaks by stabilizing a transient intermediate of the topoisomerase reaction in which the enzyme is covalently linked to the 5' terminus of a DNA duplex [8]. DNA damage induced by these cleavable complexes triggers cell death by apoptosis. Topoisomerase II inhibitors are most active in S-phase cells in which the enzyme is highly expressed [9]. Both dihydrofolate reductase that is the target of methotrexate, and thymidilate synthase that is inhibited by 5-fluorouracil are also typical S-phase enzymes. Conversely, vinca-alkaloids and taxans, that inhibit the formation of the mitotic spindle, require cell cycle progression through M-phase to be cytotoxic [10].

The vulnerability of proliferating cells to DNA damage is related to the large number of proteins that are required for cell cycle progression, each step of the process being a potential target for inhibitors. Thus, it is easily conceivable that quiescent cells arrested for a long time in the G₀/G₁ phase are protected from the toxic effects of intermittent administration of drugs whose activity depends on cell cycle progression [11,12]. However, this simple explanation does not reflect all the facets of kinetic resistance. In this paper, we review recent findings about cell cycle progress, apoptosis induction, stress defence mechanisms and cell-matrix interactions that could help to understand why cancer cells included in a tumor mass better escape drug-induced cell death than isolated cells.

Cell Cycle and Response of Cancer Cells to Chemotherapy

Understanding the mechanisms of cell cycle regulation has rapidly progressed in the ten past years [13]. In higher eukaryotes, cell cycle progression through G₁ phase (gap before DNA replication), S phase (DNA synthesis phase), G₂ phase (gap after DNA synthesis) and M phase (mitotic phase) is controlled by a series of cyclins and their partners, the cyclin-dependent kinases (CDKs). The decision to divide occurs as cells pass a restriction point late in G₁,

after which they become refractory to extracellular growth regulatory signals and progress autonomously through the S, G₂ and M-phases. Passage through the restriction point and entry into S phase is controlled by CDKs whose activity is sequentially regulated by cyclins D, E and A. CDK activity not only requires cyclin binding but also depends on both positive and negative regulatory phosphorylations [14] and can be constrained by two families of CDK inhibitors (CDKI). One family includes four so-called 'INK4 proteins', p15^{INKb}, p16^{INKa}, p18^{INKc} and p19^{INKd}, that are specific inhibitors of CDK4 and CDK6, the catalytic partners of cyclins D. Three other proteins, p21^{CIP1}, p27^{KIP1} and p57^{KIP2}, constitute the CIP/KIP family of CDKIs that inhibit cyclins D, E and A and their catalytic partners [15]. Cyclins/CDKs complexes promote cycle progression through the inactivation by phosphorylation of the retinoblastoma protein Rb and the Rb-like proteins. The hypophosphorylated Rb binds and inactivates a family of transcriptional regulators, collectively termed the E2Fs, that transactivate S-phase genes such as dihydrofolate reductase, thymidilate synthetase, ribonucleotide reductase, and thymidine kinase [16].

Many antitumor agents exert their cytotoxic activity by damaging the DNA structure. Progression of replication forks during S-phase is critical for DNA integrity [17] and the collision between a replication fork and a modified DNA structure accounts for the cytotoxic effect of cisplatin, camptothecin and alkylating agents. Similarly, DNA strand breaks induced by topoisomerase I (camptothecin) and II (etoposide, anthracyclines) inhibitors are lethal in S-phase traversing cells but are repaired in quiescent cells [18,19]. Cells respond to DNA damage by arresting cell cycle at different checkpoints and inducing transcription of genes that facilitate DNA repair. When DNA damage is correctly repaired, cells progress again through the cell cycle. In contrast, cell death ensues when the damage is irretrievable. At least two checkpoints have been clearly identified in response to DNA damage, one in the G₁-phase and the other in the G₂-phase [20]. The p53 gene is an archetypal checkpoint regulator in response to DNA damage [21]. The normally very short-lived protein p53 is not essential for cell cycle progression but accumulates in cells undergoing DNA damage and as a result brakes the cycle in G₁ to maintain genomic stability. The precise signal transduction pathway that senses DNA damage and recruits p53 has not been elucidated but is likely to include the ATM gene, a gene mutated in ataxia

telangiectasia. The gene encoding the CDK inhibitor p21^{CIP1} that functions as a transcription factor is one of the targets of p53. Combination of two molecules of p21^{CIP1} with either cyclin D1/Cdk4/PCNA or cyclin A/cdk2/PCNA or cyclin E/cdk2/PCNA complexes inhibits the phosphorylation of Rb, thereby inactivating E2Fs and preventing cells from entering into S-phase.

In almost 50% of human cancer, p53 is inactivated by mutation [22]. The loss of a functional p53 predisposes tumor cells to drug-induced gene amplification and decreases the fidelity of mitotic transmission by altering the duplication of the centrosomes that normally begins at the G₁-S boundary. The resulting genetic instability leads to changes in chromosome number and increases the probability that such cells evolve toward malignancy, escape immune surveillance, tolerate hypoxia and become angiogenic and drug resistant. The G₂-M transition is regulated by the cyclin B/p34^{cdc2} kinase complex which remains inactive during S and G₂ phase as a result of phosphorylation at Tyr¹⁵ by the wee 1 kinase [23]. In cells lacking a functional p53, the G₂-M checkpoint remains the only safeguard to avoid transmission of damaged DNA to daughter cells [24, 25]. We have observed that the resistance of p53-negative leukemic cell lines to etoposide-induced apoptosis increased with their ability to arrest cell cycle progression in G₂ phase in response to similar levels of DNA damage induced by the drug [26]. In human tumors, hypoxia could contribute to the slow progression of tumor cells in the cell cycle by activating p53 [27]. Although the quiescent cells could be less sensitive to cytotoxic drugs as a consequence of a longer stay in the G₁ and G₂ phase of the cell cycle, increased DNA repair in these low cycling cells could also play a role in their intrinsic resistance to anticancer drugs.

Cell Cycle and Cell Death by Apoptosis

Since the processes of cell loss and cell gain must be homeostatically balanced in order to maintain the complex dynamic architecture of tissues and allow adaptation to changing circumstances, several links have been suggested between cell proliferation and cell death. Some of the morphological changes observed in cells undergoing apoptosis are reminiscent of mitosis. Therefore, apoptosis has been described as an abortive mitosis [28]. Both mitotic and apoptotic cells condense their chromatin and disassemble the nuclear lamina, release substrate attachments, become

more rounded and shrink. However, the similarities end there. Unlike mitotic cells, apoptotic cells do not assemble a mitotic spindle, they display rapid cytoplasmic blebbing, they express on their surface novel determinants that trigger phagocytosis and, in most cases, they cleave their DNA into approximately 200 bp fragments. The biochemical changes that occur during each process is strikingly different. For example, disassembling of the nuclear lamina results from the p34^{cdc2}-mediated phosphorylation of lamins during mitosis and caspase-mediated cleavage of the lamins during apoptosis [29].

Mitotic regulators are involved in some apoptotic pathways. Cisplatin-induced apoptosis on immature rat thymocytes depends on the presence of cells in S and G₂-M phases of the cell cycle [30]. Cell death triggered by the cytotoxic T cell or natural killer cell granule protease granzyme B involves activation of cyclin A-p34^{cdc2} kinase in target cells. Overexpression of the wee1 kinase rescues granzyme-B induced cell death by inhibiting p34^{cdc2} [31]. Ectopic expression of the CDK PITSLRE protein kinase induces telophase arrest and apoptosis. On the other hand, Fas-mediated killing pathway is associated with elevation of PITSLRE activity [32]. Activation of mitotic regulators is not a universal requirement for apoptosis since p34^{cdc2} is not activated during apoptosis of fibroblasts following serum deprivation but several recent reports involved these regulators in drug-induced cell death. First, treatment of the human leukemic HL-60 cells with the topoisomerase I camptothecin induced unscheduled activation of cyclin B1/p34^{cdc2} kinase [33]. Secondly, elevation of p34^{cdc2}, CDK2 and cyclin A was reported in Hela cells treated with a variety of apoptosis inducers [34]. Finally, by the use of a histone H1 kinase assay, we have evidenced a low kinase activity of the cyclin B/p34^{cdc2} in HT29 hyperconfluent cells that are resistant to drug-induced cell death (unpublished results).

More generally, apoptosis is proposed to result from incompatible signals for proliferation and cell cycle arrest [35]. Several oncogenic lesions that generate uncontrolled cell proliferation also promote cell death. The adenovirus E1A gene drives the host cell proliferation necessary for virus replication but it also potentially triggers apoptosis when mutant viruses that lack a functional E1B gene infect host cells [36]. The mammalian cellular protein c-Myc exhibits a similar duality of function to E1A. Interestingly, the anti-apoptotic protein Bcl-2 suppresses only the apoptotic actions of both E1A and c-Myc, leaving their abilities

to promote cell proliferation unaffected [37]. A major coordinator of both cell proliferation and apoptosis is Rb. One of the functions of p105^{Rb} is to suppress the action of the G₁-specific E2F transcription factors which coordinate expression of the late G₁ and the early S phase genes required for cell cycle progression. Deregulated expression of E2F transcription factors induces a phenotype analogous to E1A, namely induction of both proliferation and apoptosis. p105^{Rb} might prevent apoptosis by inhibiting S phase entry, thus preventing unscheduled DNA synthesis. Inappropriate c-Myc expression can override the growth suppressive effects of p105^{Rb}. Thus, c-Myc could push a cell into a premature cell cycle, resulting in some internal conflict which triggers apoptosis. Ectopic expression of c-Myc is lethal only under conditions in which serum is reduced, suggesting that activation of c-Myc in the absence of complementary cytokines leads to dysfunctional cell cycle progression resulting in apoptosis. However, induction of apoptosis by c-Myc is independent of cell cycle position and the mechanisms which explain apoptosis induction by c-Myc remain hypothetical [38].

Whether or not apoptosis occurs as a consequence of a defective cell cycle, it is clear that alteration of genomic integrity is an efficient trigger of apoptosis and virtually all DNA damaging agents kill cells by inducing apoptosis. Consequently, cells that can suppress this suicide would have gained an entirely new kind of drug resistance. In tumors containing such cells, genotoxic agents would presumably act as effective mutagens, driving the genetic diversification and compounding the problems of future therapy. We have described the role of p53 in DNA damage-induced transient cell growth arrest that reflects the time used by the cell to repair the damaged DNA template before replication and mitotic chromosome segregation [26]. The relationships between p53 gene expression and DNA damage-induced apoptosis is less clear. Wild-type p53 is necessary for the rapid induction of high levels of apoptosis in medulloblastoma cell lines following irradiation while glioblastoma cell lines undergo only p53-independent apoptosis in response to radiation [39]. Moreover, in these cells, apoptosis is delayed and occurs only at low levels after high doses of radiations. The p53 protein transcriptionally activates the expression of a number of well-defined target genes including the pro-apoptotic gene bax. However, p53-dependent thymic apoptosis is normal in bax knock-out mice [35]. A great deal of information is still needed to define the role of p53

in determining cell cycle arrest versus apoptosis after DNA damage. This activity is likely to be of great importance for cancer therapy since p53 appears capable of modulating apoptosis from an extremely diverse set of cellular, pharmacologic, or environmental stresses.

Cell Adhesion and Apoptosis

Intrinsic kinetic resistance to cytotoxic drugs can be reproduced by growing the cells as multicellular spheroids [40,41] or as hyperconfluent cultures [42]. The concept of 'acquired multicellular resistance' was developed to explain why cancer cells growing as spheroids are much more resistant to 4-hydroperoxycyclophosphamide than the same cells growing as monolayer. Hyaluronidase, an anti-adhesive enzyme that disrupts spheroids, was shown to sensitize tumor cells to anticancer agents either *in vitro* or *in vivo* [43,44]. A key regulatory protein in this process might be p27Kip1. This CDK inhibitor appears to be the most directly involved regulator of the G₁-S restriction point control and to regulate both cell size and cell number [45]. In quiescent cells, p27Kip1 levels are high, but once cells enter the cycle, p27 level falls. The cellular level of p27Kip1 is mainly controlled by posttranslational mechanisms [46] and decreased p27Kip1 level has recently been shown to be a negative prognostic factor in various human solid tumors [47]. Conversely, p27Kip1 level, which is upregulated by intercellular contacts in colon cancer cell spheroids, has been proposed to be the mediator of contact inhibition-associated multicellular resistance [48]. In accordance, we have recently observed that p27Kip1 level increased in parallel to growth inhibition and resistance to doxorubicin in HT29 human colon cancer cells grown either as non confluent or confluent populations (unpublished results). The influence of cell-cell and cell-matrix interactions upon proliferation and differentiation of normal epithelial cells is well established. Intestine cells while differentiating, proliferate in the depth of the intestinal crypts, then progress along the crypt, tightly bound to the neighbouring cells. At the end of their migration, intestine cells detach from the epithelial sheet and die. Colon cancer cells probably retain some of the characteristics of normal cells, particularly the role of cell to cell contact in the regulation of cell proliferation and survival. Colon cancer cells grown as spheroids are disaggregated by calcium removal from the culture medium. When calcium-induced reaggregation is inhibited by

a monoclonal antibody against an $\alpha v \beta 3$ integrin epitope, cells enter in a rapid apoptotic process associated to a relocation of p53 into the nucleus [49]. Analogous, adhesion of mammary epithelial cells to the extracellular matrix through integrins transmits signals that suppress apoptosis, by preventing the activation of intracellular proteases related to the interleukin-1 β converting enzyme, the so-called 'caspases' [50]. In absence of cell-matrix contact, epithelial cells arrest in G₁ and are committed to apoptosis, a phenomenon that is associated to the caspase-mediated cleavage of Rb and is inhibited by the anti-apoptotic bcl-2 gene product [51]. Although some mechanistical information now has emerged as to how receptors for the extracellular matrix (integrins) may alter cell cycle activity [52] or apoptosis [53] in response to their ligands, very little is known about signalling from cell-cell interactions. Different cell types, including various cancer cell lines, respond differentially to loss of cell-cell contacts with respect to apoptosis, and it has been suggested that this may be an indirect effect of cell cycle activity [53]. While nontransformed cells seem to tolerate loss of extracellular matrix contact poorly [54,55], transformed cells may be able to utilize intercellular adhesion receptors as a survival system. We have preliminary data that suggest that E-cadherin and integrin systems may cooperate in intercellular adhesion to ensure proper contact and survival of human colon cancer cells. These results will aid in the understanding of how cell-cell contacts may modulate cell cycle activity, resistance to cytotoxic drugs and apoptosis.

Drug Induced Cell Death and the Effector Phase of Apoptosis

Whereas the initiation phase of apoptosis depends on the type of cell death-inducing stimulus, i.e. whether it damages DNA or not, the effector phase is common to all apoptotic processes. The family of Bcl-2-related proteins constitutes one of the most relevant classes of proteins that regulate this effector stage. The Bcl-2-related proteins can either antagonize (Bcl-2, Bcl-X_L, Bcl-w, Bfl-1, Bcl-2L1, Mcl-1 and A1) or promote (Bax, Bcl-X_S, Bak, Bad, Bid, Bik and Hrk) cell death. Most of these proteins have to localize into the mitochondria to regulate apoptosis that depends on the ratio of death antagonists to agonists. This death antagonist-agonist rheostat is mediated by competitive dimerization between selective pairs of antagonists and agonists as

well as posttranslational modifications and protease-mediated degradation of Bcl-2-related proteins. Signal transduction via growth factor receptors and kinase cascades can influence the phosphorylation states of Bcl-2-related proteins and can influence the subcellular distribution of proteins that interact with Bcl-2 such as the Raf-1 kinase. The three-dimensional structure of Bcl-X_L monomer in the absence of its carboxy-terminal signal anchor sequence suggests a similarity to the pore-forming domains of bacterial toxins. This protein can insert into lipid bilayers and form an ion-conduction channel. However, it remains to be demonstrated whether the death-regulating functions of Bcl-2-related proteins involves these channel properties. One of the main functions of Bcl-2 might be a membrane-stabilizing effect that prevents the release of apoptogenic proteins from mitochondria [56].

Given the functional importance of Bcl-2-related proteins in apoptosis control, they constitute a potential target for the induction of apoptosis in tumor cells. Overexpression of Bcl-2 and Bcl-X_L in normal and neoplastic cells delays apoptosis induced by radiations, glucocorticoids and many chemotherapeutic agents [57]. Induction of Bax in p53-deficient cells sensitizes them to the cytotoxic effect of taxol, vincristine and doxorubicin [58] but does not restore the apoptotic response to DNA damaging agents in thymocytes from p53 $-/-$ mice, indicating that p53 is required to induce or activate factors other than Bax [59]. Interestingly, cytotoxic drugs acting on microtubules such as taxans inactivate Bcl-2 via its hyperphosphorylation during the G₂-M phase.

A key regulator of apoptosis is the Fas receptor/Fas ligand system. We have observed that treatment of tumor cells with several cytotoxic drugs enhance the expression of a functional Fas receptor [60]. Bcl-2 failed to protect isolated mitochondria against the release of apoptogenic factors induced by cytosols from Fas-stimulated cells, suggesting that the Fas-mediated pathway could be used to bypass the Bcl-2-mediated resistance to cytotoxic drugs [61]. Both increased Bcl-2 expression and decreased expression of Bax were related with poor response to chemotherapy in some human tumors [62,63]. However, delayed apoptosis induced by Bcl-2 does not always correlate with increased drug-resistance [64]. Using a tetracycline-regulated expression system, overexpression of Bcl-2 was shown to delay apoptosis induction by colcemid and trimetrexate without increasing clonogenic survival [65].

Heat shock proteins (HSP) constitute another family of molecules that can modulate cell sensitivity to apoptosis. Increased expression of HSPs 27, 70 and 90 renders tumor cells more resistant to various cytotoxic agents including camptothecin, etoposide and actinomycin D [66–70]. Transfection of human cancer cell lines with hsp27 increased cell resistance to cisplatin, doxorubicin and etoposide but not to 5-fluorouracil nor to nitrosoureas [71–73]. HSP27, whose level increases in colon cancer cells as they reach confluence, could protect from cell death by decreasing the level of intracellular reactive oxygen species [74].

In conclusion, the effect of cytotoxic drugs used for human cancer chemotherapy can involve induction of apoptosis pathways, in addition to their direct toxic effect on DNA or metabolism. Sensitivity or resistance of tumor cells to these drugs can be modulated by the complex molecular systems that induce, transduce or regulate the apoptotic signal in the cancer cell.

Circumvention of the Kinetic Resistance

The goal of this review was to attract attention of oncologists on a predominant but yet understudied mechanism of resistance in solid tumors. Drawing up the inventory of this complex epigenetic phenomenon could permit to propose some solutions to circumvent kinetic resistance.

The first challenge is the choice of anticancer drugs that remain toxic on quiescent cells. Most available anticancer agents have been selected using panels of cancer cell lines or animal models with a high growing rate in order to save time and to screen a large broad of natural or synthetic substances. In contrast, very few studies have been done on the cytotoxic effects of common anticancer drugs on spheroids or hyperconfluent cell cultures. Using the HT29 human colon cancer cell line that grow easily as adherent monolayers, we have designed a specific cytotoxic assay for hyperconfluent cells. These cells are partly contact-inhibited at confluence, a situation presumed to reproduce the environment inside a solid tumor and they display a so called ‘Confluence Dependent Resistance’ to anthracyclines and other anticancer agents [42,75]. HT29 cells were seeded at high density 15 days before assay then treated by short (4 h) or prolonged (24 h) exposure time to various anticancer drugs. Cells were cultured for 15 additional days in drug free medium to let cells die by apoptosis or mitotic death, and then analyzed by the use of an MTT assay to determine if

the residual ghostly cells were metabolically active. In contrast to classical clonogenic assays, this assay does not require a trypsin-mediated detachment of treated cells that could restore cell cycle and increase drug-induced cytotoxicity.

Results shown in Figure 1 confirm the deleterious effect of confluence on most classical anticancer drugs. Most drugs including 5 fluorouracil and taxanes were unable to kill the entire cell population, even at a high and sustained concentration. Conversely, some agents such as cisplatin, mitomycin C and thiotepa kept some activity on hyperconfluent cells. Increased exposure time improved drug efficacy, suggesting an advantage of continuous treatment of quiescent cells in accordance with empirical observations from clinical oncologists. For many drugs, we observed an important gap, most often higher than one logarithm, between the concentrations necessary for decreasing cell survival (partial response) and those which produce a total cell killing (complete response). The most suitable drugs for solid tumors might be those with the smaller difference in cytotoxicity on confluent compared to non confluent cells. Conversely, drugs whose cytotoxicity is limited by a plateau phase, eg presence of persisting survival cells despite a more than one log increase of drug concentration, should not be used alone for the treatment of solid tumors. Increasing drug concentration obviously increased cell killing by many drugs, a concept that sustained intensification strategies in chemotherapy. Our observations on confluent cells shows that drugs selected for these intensifications should preferentially include those with a curve of cytotoxicity that rapidly crosses the baseline. Our results are consistent with those of Frankel et al. which observed an abrogation of paclitaxel-induced apoptosis in human ovarian cancer cells grown as multicellular spheroids; in contrast, cisplatin retains the same cytotoxic activity on monolayer or on spheroid cultures [76]. Interestingly, cytotoxicity of a single dose irradiation was independent of the degree of confluence in the cancer cell population.

Polychemotherapy is more active than monotherapy although the biochemical bases for this concept are not fully understood. Current polychemotherapy regimens result from long trial-and-error studies based on empirical or theoretical considerations. Sequence and time between drugs are important parameters for the efficiency of these regimens. However, positive or negative interactions between two or more drugs, which have been studied *in vitro* on a limited number of cell models, are not directly trans-

Figure 1a. HT29 human colon cancer cells (10^5 /well) were seeded in 96 well tissue culture plates and cultivated for 15 days to obtain a confluent culture. Non confluent culture was obtained by seeding 10^4 cells/well for 48 h before treatment. Cells were exposed for 3 or 24 h to drugs, then cultured again for 15 additional days in drug free medium. Irradiation was performed one time on confluent or non confluent cells. Cell survival was measured by a MTT colorimetric assay.

possible in clinical practice. One of the approaches to enhance the efficacy of anticancer agents on quiescent cells is to push them from the G_0 - G_1 to the S and the G_2 -M phases of the cell cycle. Treatment of quiescent cells with low doses of DNA damaging agents induces such a progression through S and G_2 , thereby increasing topoisomerase II cellular content and cell sensitivity to its inhibitors [77]. Host cells remain protected from low doses of DNA damaging agents by the wild-type p53 that arrests them at the restriction point while p53-deficient cancer cells accumulate in S and G_2 phases where they are preferentially killed by cell cycle specific agents. Accordingly, cisplatin, mitomycin and melphalan increase the proportion of con-

fluent Caco2 colon cancer cells that progress through the S and G_2 phases and sensitize them to the cytotoxic effect of doxorubicin [78]. Combination of the S/ G_2 recruitment and verapamil as an MDR-reversing agent results in an almost complete circumvention of the high intrinsic resistance of confluent Caco2 cells to anthracyclines. A similar observation was made by Toffoli et al. which demonstrated that the effect of verapamil on doxorubicin cytotoxicity is maximal when inhibition of Pgp activity occurs during the S/ G_2 M phases [79]. In our opinion, combinations of classical and new anticancer agents should be evaluated on quiescent cancer cells to identify synergistic effects that might be tested in clinical trials. An other strategy

Figure 1b.

is the pharmacological inhibition of checkpoint functions which facilitate DNA repair; G2 arrest induced by DNA-damaging agents is inhibited by UCN-01, a protein kinase C inhibitor, resulting in an enhanced cytotoxicity of cisplatin and gamma radiation [80]. The same result was obtained with caffeine or pentoxifylline which enhance the sensitivity of cancer cells to anticancer agents by overriding the G2/M delay [25,81–83]. However it remains to check that this strategy works on confluent cancer cells that are mainly arrested in the G0/G1 phases.

Recent findings on cell cycle and cell death have suggested new strategies to circumvent kinetic resistance of tumor cells to anticancer drugs. Proteins that brake the cell cycle in response to stress, genomic damage or extracellular signals such as p53, p21^{CIP1}

and p27^{KIP1} as well as those that inhibit the effector stage of apoptosis such as Bcl-2 and Bcl-XL may be viewed as proteins that decrease cell sensitivity to multiple cytotoxic drugs [84–88]. Strategies aiming either to downregulate the expression of these proteins, for example by using antisense nucleotides, or to upregulate their antagonists, for example by using gene therapy, or to use alternative pathways, for example the Fas-mediated pathway [60], may improve the efficacy of anticancer therapy on solid tumors.

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