Malignant neoplasms consist of heterogeneous cell populations and their cellular elements proliferate asynchronously. Since the tumor cells of various cell cycle phases respond differently to many chemotherapeutic drugs, attempts at synchronization seemed to be a promising way to achieve a more powerful antineoplastic effect. Mainly based on in vitro data, it was shown that numerous compounds, including hormones, were able to arrest the cell cycle in different phases, and some of them also induced apoptotic cell death. The better understanding of the molecular mechanisms of cell cycle control has brought the cyclin-dependent kinases into focus and hundreds of compounds have been synthesized in order to regulate malignant cells at their checkpoints, especially at G1 progression. Some of these compounds have been found to be effective not only in vitro, but also in in vivo experiments, and they were further evaluated in Phase I – II clinical trials. Generally speaking, these studies have yielded modest, although potentially promising, results, but the adverse effects sometimes restricted the applicability of the products. Nevertheless, extended studies in cancer patients are under way. Moreover, after encouraging preclinical investigations, the combination of cell cycle regulators with different cytostatic drugs may offer a novel therapeutic alternative in the field of oncology.

During the evolution of malignant neoplasms, genetic instability, random mutations, abnormal mitoses and micro-environmental effects result in a profoundly heterogeneous cell population within the individual tumor. Significant intratumoral DNA heterogeneity has long been recognized in different malignancies, e.g. in colorectal, renal, prostatic, breast, ovarian, head and neck cancers, or gliomas (1-10). Moreover, the degree of heterogeneity is increased with the tumor stage and grade (2, 10). Regarding ploidy analysis, different regions of a given tumor may display evenly DNA diploid, DNA diploid + aneuploid or various aneuploid stemlines (3, 4, 6). Similarly, the proportion of the S-phase fraction (SPF) is also inhomogeneous: in breast cancer samples it can vary between 2 – 32 % (5, 7) and in ovarian carcinomas between 0 – 28 % (8), depending on which part of the tumor is analyzed. In a Swedish study, SPF heterogeneity reached as high as 71% in breast carcinomas (6). Proliferation rate, as measured immunohistochemically by means of PCNA, has also proved to be considerably variable, ranging from 0.7 to 45% in different areas of breast cancer samples, the PCNA score being higher in peripheral regions than in the central areas (5). In xenotransplanted human tumors, substantial heterogeneity in the cell cycle phases could also be observed (11, 12). Thus, genetic and kinetic heterogeneities may cause significant errors in an attempt to predict the clinical prognosis of a given tumor when it is based on a single sample. In addition, there may be a clinical impact because malignant cells in various cell cycle phases may respond differently to antineoplastic treatments and a number of chemotherapeutic drugs exhibit a phase-specificity.

Early studies with hydroxyurea revealed that it is active as a tumor-inhibiting drug at the G1-S boundary of the cell cycle (13), and its phase-specificity was also demonstrated in animal experiments (14). Using CEM lymphoma cells, flow cytometric studies have shown a cell cycle arrest in early S-phase, but later than the aphidicolin arrest point (15). Doxorubicin (adriamycin) predominantly induces G2 arrest (16, 17), while 5-fluorouracil (5-FU) results in an increase in G1-S-phase tumor cells accompanied by augmentation of the p21 expression and a striking cyclin E up-regulation (18). Etoposide, cisplatin or phleomycin D1 were found to block the S-phase entry, which was sustained for up to two weeks.
proved to synchronize the cell cycle both pancreatic cell lines (27). Aphidicolin, a potent mycotoxin, arrest not only porcine fetal fibroblasts (26), but also human can be exploited only with no genotoxic or cytotoxic effects (25), but this feature has no clinical connection. Similarly, dimethyl sulfoxide (DMSO) can easily be achieved and studied in cultured cells, therefore most of the studies have been performed in vitro. Many different pharmacological methods have been established. The classical method is serum deprivation but, of course, it does not work in all cases. For example, several flavonoids may also induce arrest in cultured cell lines, but the site of checkpoints varies with the flavonoids applied or with the cell types (23). Carotenoids, especially lycopene, were also reported to inhibit the cell growth of human prostatic carcinoma in vitro and, during this process, the number of cells in G2-M-phase accumulated and there was a decrease of the S-phase cells (24).

All these data indicate that various antitumor agents have points of attack at different cell cycle phases. Theoretically, if the cells in a malignant tumor displaying various stages of the cycle were in the same phase, selective, phase-specific cytostatics could be administered and their therapeutic effect would be more marked. Attempts at synchronization of tumor cells are based on this assumption.

**Synchronization of proliferating cells**

The process by which proliferating cells are arrested in specific phases of the cell cycle is termed synchronization. It can easily be achieved and studied in cultured cells, therefore most of the studies have been performed in vitro. Many different pharmacological methods have been established. The classical method is serum deprivation but, of course, it has no clinical connection. Similarly, dimethyl sulfoxide (DMSO) is also a powerful inducer of G1 synchronization with no genotoxic or cytotoxic effects (25), but this feature can be exploited only in vitro. Butyrolactone-I was shown to arrest not only porcine fetal fibroblasts (26), but also human pancreatic cell lines (27). Aphanthocin, a potent mycotoxin, proved to synchronize the cell cycle both in vitro and in vivo (28), the majority of cells being accumulated at the G1-S transition (26). Staurosporine also reversibly inhibits lymphoma cells acting in early G1 (15).

Among the cell cycle regulators mimose, a nonprotein amino acid derived from Mimosa and Leucaena plants, has long been recognized as a synchronizing agent. On addition to asynchronously growing cultures (human lymphoblastoid, erythroleukemia, promyeloid leukemia, HeLa, EJ30 cells), it inhibited the cell cycle at late G1, time- and dose-dependently (29-33). The exact mechanism by which mimose exerts its blocking effect is still incompletely understood, but multiple targets seem to be important: interference with the synthesis of histone H1 kinase, a key enzyme that is required for cell cycle progression (34), up-regulation of p27(Kip1) protein levels (35), specific inhibition of cyclin D1 expression (36), as well as its iron chelating effect have been reported (37).

It is of special interest that the synchronization effect can also be achieved by hormonal manipulations. It was reported, more than a decade ago, that glycoconjugates were reversibly induced an early G1 block in a hepatoma cell line, preventing the cells from entering the S-phase, accompanied by the elevated expression of c-jun transcripts (38). Antiestrogens can also modulate the cell cycle. Tamoxifen (and related compounds) gives rise to an accumulation of human breast cancer cells in the G1-phase; more than 90 % of cells were in G1 72-96 hours after adding Tamoxifen to the culture and the arrested cells retained their viability and responsiveness to estrogen (39-41). These effects of antiestrogens depend strongly on the presence of specific estrogen receptors (42). Somatostatin (SS) and its analogs also initiate antiproliferative signals in various endocrine and non-endocrine tumors, and the ligand-activated SS-receptors led to G1 arrest in vitro through up-regulation of the cyclin-dependent kinase inhibitor p27(Kip1) with concomitant accumulation and hypophosphorylation of Rb-protein (43, 44). The synthetic SS-analog octreotide was shown to exert a strong inhibitory action on cerulin-induced cyclin E expression (45). A new, synthetic heptapeptide SS-analog (TTT232) irreversibly blocks the cell cycle G1-S transition in human epidermoid carcinoma (A431) cells (46).

**From synchronization to the checkpoint-regulators**

Cell cycle regulators have attracted much attention, both in the field of cancer research and in treatment modalities. The cell cycle is mainly regulated through the cyclin-dependent kinases (CDKs) and cyclin complexes. Their deregulation often leads to development of malignant tumors, therefore it is not surprising that profound alterations are demonstrated in most human cancers. Hence, in the last decade, the "checkpoint-regulator" CDK-inhibitors have attracted particular interest and many new, promising molecules have been synthesized and tested. These studies have led to a better understanding of the molecular mechanisms of cell cycle control in malignant tumors and raised the prospect of specific molecule-targeted therapies.

On treatment of human pancreatic cancer cells with butyrolactone-I, the phosphorylation of RB-protein and the cyclin A expressions were significantly inhibited, and apoptotic cell death was detected accompanied by bel-2 down-regulation (27). As for staurosporine, the primary cellular response to administration of this compound was found to be the accumulation of p27kip1 (47), while CDK2
activity was suppressed. The latter effect was mainly due to rapidly increased levels of p21 and p27 proteins that inhibited CDK2 activity upon binding (48). Although the precise molecular mechanisms by which mimosine induces G1 arrest is still being unfolded, it seems to act at multiple points by regulating the expression of cyclins and the activities of CDKs. It inhibited cyclin D1-associated kinase activities without affecting gene transcription directly, and induced p21^{cip1} expression (independently of p53 status) and p27^{kip1} in non-small cell lung cancer cell lines. Unfortunately, one cannot generalize about these effects, because the results were cell line-dependent (36). The effect on the p21 pathway, however, seems to be more complex, because there are experimental data that continuous elevation in both p53 and p21 protein levels were detected over 48 hours after cells re-entered the cycle following synchronization (49).

Some compounds have been found effective not only in vitro but also in vivo. Olomoucine, a purine analog with a CDK1 inhibitory action (50), induced rapid and marked apoptosis in a dog with metastatic malignant melanoma after i.v. treatment for a week (51). Mimosine was also reported to significantly suppress human lung cancer growth xenografted into nude mice: 45-55% reduction of tumor volume was observed, accompanied by reduction of immunohistochemically-detected cyclin D1 and by induction of p21^{WAF1}. Moreover, TUNEL-analysis has revealed an increase in the apoptotic index of these xenografts (52). Our results also have shown that this plant amino acid can be applied in vivo without any measurable toxicity: when immunosuppressed mice bearing human pancreatic carcinoma xenografts were treated with i.p. mimosine a decreased tumor growth was achieved, and flow cytometry revealed a highly significant apoptosis (53). The Ki-67 index was also found to be significantly ($p<0.0001$) decreased indicating that, in addition to the in vitro effects, mimosine also acts as an antiproliferative agent in vivo.

Clinical applications

Without relevant clinical applicability, cell cycle blocking molecules would just remain within the confines of laboratories. Promising preclinical studies, however, have raised the possibility that various checkpoint regulators that affect G1 progression and/or G1-S transition might be suitable targets for cancer therapy (54, 55). Particularly the inhibition of CDKs seems to be the most productive strategy and hundreds of such compounds have been synthesized. Unfortunately, there is no individually selective CDK-inhibitor, but semi-selective agents (such as olomoucine), pan-CDK inhibitors (e.g. flavopiridol) or non-specific protein kinase groups (e.g. staurosporine) are worth investigating. Several Phase I and II clinical trials are currently under way, suggesting that this therapeutic approach could be justifiable in the treatment of various malignant tumors. Excellent reviews are available with these results (56, 57).

Flavopiridol, a synthetic flavonoid which inhibits several CDKs by competing with ATP, decreases the levels of cyclin D1 and D3, shows anti-angiogenic effect and induces apoptosis in vitro and in xenograft systems (58, 59), was the first such a compound to enter the clinic. Accumulated data show that this compound does have an antitumor effect on different malignancies with various therapeutic protocols; disease stabilization, minor response, partial response or even (occasionally) complete response could be achieved with mostly reversible side-effects (diarrhea, hypotension, proinflammatory syndrome, neutropenia, arterial and venous thromboses) (see 56, 57). Conversely, however, no clinical response was demonstrated in refractory mantle zone lymphoma patients who had received a prior chemotherapy regimen and 1 out of 10 patients developed grade III-IV non-hematological toxicity (60).

Staurosporine, which is a promiscuous protein kinase C inhibitor, per se has not been applied in clinical trials, but its derivate, 7-hydroxystaurosporine (UCN-01), is being tested with modest results (reviewed in 56, 57). In one patient suffering from malignant melanoma partial response was observed, and an anaplastic large cell lymphoma patient is disease-free after 4 years of introduction of the therapy (57). Side-effects were vomiting, hypoglycemia, hypotension and pulmonary toxicity. In another Phase II study, 21 renal cell carcinoma patients were treated with UCN-01, but no objective response was demonstrated. Although the therapy was well-tolerated, the time to disease progression proved to be about 3 months (61).

Proteosome inhibitors interfere with the degradation of CKIs (p21, p27) and may lead to stabilization of both CKI and cyclins. To date, only a single agent (bortezomib, Velcade) has been approved to enter clinical trials. Bortezomib showed antitumor activity in various hematological malignancies (62) and solid tumors (63) with reversible, but sometimes serious, side-effects. In a multiple myeloma patient, a complete response and in patients with mantle zone or follicular lymphoma, shrinkage of the tumor was noted (64). Phase III trials of this compound are under way.

E7070 is a sulfonamide-related compound that induces arrest at the G1-S boundary and inactivates the cyclin E/cdk2 complex. Administration of this drug to tumors patients has yielded a limited antineoplastic effect: less than one-third of patients displayed temporary stabilization of their diseases, occasional partial response was observed and the dose-limiting toxicities were mainly hematological (65, 66).

Depsipptide (FR901228) is a histone deacetylase inhibitor that was shown to produce mitotic arrest with G2-M accumulation in different cell lines (67). Phase I trials have demonstrated some antitumor activity of this
compound: in 1 out of 37 patients with refractory neoplasms a partial response was observed (68), while the therapeutic results were more promising in T-cell lymphomas; one patient with peripheral T-cell lymphoma having a complete response and 3 cutaneous lymphomas exhibiting a partial response (69). In chronic lymphoid leukemia or acute myeloid leukemia patients, however, no objective responses were noted (70). At present, Phase II studies are under way.

Another histone deacetylase inhibitor, a synthetic benzamide derivative (MS-27-275), was also tested in vitro and in vivo systems and Phase I studies are currently under way. MS-27-275 resulted in a shift in cell cycle (decrease of S-phase cells, increase of G1), induced p21WAF1/cip1, and it had a marked antineoplastic activity in xenografts of various tumors (71, 72).

Combination treatment modalities

Unfortunately, malignant neoplasms frequently acquire resistance to the administered chemotherapeutic drugs during treatment. Multiple points of attack may result in a more powerful tumor-killing effect, may overcome the resistance and the individual doses could be decreased, minimizing the – sometimes serious – toxicities. Therefore, the combination of different drugs is a well established regimen in oncology. Accumulating data suggest that a combination of cell cycle regulators with different cytostatic drugs may be an alternative of choice.

In vitro studies have revealed that butyrolactone-I enhanced human colorectal carcinoma cell (DLD1) survival after doxorubicin treatment, but, unfortunately, it also blocks the process of apoptosis (73). Aphidicolin was also checked in vitro, and advantageous effects were detected: it enhanced etoposide cytotoxicity on human ovarian carcinoma cells (74), increased significantly the sensitivity of leukemia cells to cytosine arabinoside (particularly in acute myeloid leukemia) (75), and it was found to act synergistically with vincristine and doxorubicin against neuroblastoma cell lines (76). However, no potentiating effect was observed on cisplatin-resistant colonic or lung cancer cell lines (77) that might suggest a tumor-specificity.

Staurosporine and its derivatives have also been studied in combination regimens. After pretreatment with staurosporine, breast cancer cells were selectively killed by doxorubicin and camptothecin, whereas normal mammary epithel cells resumed their proliferation after the cytostatic drugs were washed out (78). 7-Hydroxystaurosporine (UCN-01) proved effective not only in vitro but also in vivo experiments. Theoretically, chemotherapy should be more effective if the tumor cells (or the majority of them) were synchronized, because many chemotherapeutic drugs exhibit a phase-specificity. Recognition of the molecular checkpoints of the cell cycle has led to a novel antineoplastic strategy: inhibition of the cyclin- dependent kinases. Many cell cycle regulators have been synthesized and some of them are effective not only in vitro but also in vivo experiments. Based on encouraging experimental studies, clinical trials are currently under way with these compounds, mainly in

Tamoxifen, this combination inhibited the cell growth synergistically and in xenografts exhibited superior antitumor effects. The results of ongoing clinical trials will show the real place of UCN-01 in practice.

Experimental and clinical studies have also been performed applying flavopiridol in combination. In addition to its radiosensitivity-enhancing effect alone or with docetaxel (82, 83), it enhanced the apoptotic rate of docetaxel in human gastric cancer in vitro, and markedly interfered with tumor growth in gastric cancer xenografts (84). The latter results seem to be meaningful, because carcinomas of the stomach are rarely sensitive to docetaxel. However, combination therapy in patients with metastatic breast cancer (n=6) failed, because dose-limiting neutropenia developed, although two disease stabilizations and one partial response were observed (85). Flavopiridol was also evaluated in a Phase I study in patients with advanced solid tumors (esophagus, lung, prostate carcinomas) following paclitaxel infusions, and clinical activities were observed. Despite neutropenia and pulmonary toxicity at higher doses, the authors recommended Phase II trials with lower dosage (86).

Earlier, it was mentioned that the proteosome inhibitor bortezomib given alone showed antitumor activity in various malignancies (62, 64). Experimental data have also shown its effectiveness when combined with cytostatic drugs. Using A549 non-small cell lung cancer cells, bortezomib plus a gemcitabine/carboplatin combination proved to be schedule-dependent: when the proteosome inhibitor was applied first, apoptotic activity was diminished, but simultaneous administration resulted in an increased apoptotic ratio (87). In another study, bortezomib in combination with gemcitabine enhanced p53 and p21 expression, induced apoptosis, destroyed 80% of tumor cells, and the A549 xenografts grew significantly slower in the combination group than in animals given gemcitabine alone (88). The same group has reported the results of the combination of two cell cycle regulators in lung cancer cells: bortezomib + a histone deacetylase inhibitor synergistically increased apoptosis (89). A similar synergistic effect was also observed in vitro and in vivo using bladder carcinoma cells and xenografts grown in nude mice (90).

Conclusion

Theoretically, chemotherapy should be more effective if the tumor cells (or the majority of them) were synchronized, because many chemotherapeutic drugs exhibit a phase-specificity. Recognition of the molecular checkpoints of the cell cycle has led to a novel antineoplastic strategy: inhibition of the cyclin- dependent kinases. Many cell cycle regulators have been synthesized and some of them are effective not only in vitro but also in vivo experiments. Based on encouraging experimental studies, clinical trials are currently under way with these compounds, mainly in
Phase I or II. Administration of CDK inhibitors alone has rarely yielded complete remission of malignant tumors, though it is possible that combination with traditional cytostatic drugs may enhance their tumor-killing effects.

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References


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