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Sensitization of Tumor Cells to Ribotoxic Stress-induced Apoptotic Cell Death: A New Therapeutic Strategy

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Abstract
We describe a procedure that sensitizes chemotherapeutic and tumor necrosis factor-resistant human tumor cell populations in vitro and in nude mouse transplants to the immediate triggering of high rates of cell death by anisomycin, an agent causing activation of stress-activated protein kinases [SAPKs, as defined by P. Cohen (Trends Cell Biol., 7: 353–361, 1997)] including p38/RK and c-jun NH2-terminal kinase homologues, following its binding to ribosomal 28S RNA (M. S. Iordanov et al., Mol. Cell. Biol., 17: 3373–3381, 1997). Sensitization is effected by successive application of an inhibitor of histone deacetylation (trichostatin A, butyrate) and of flavopiridol, known as an inhibitor of cyclin dependent kinases and evaluated presently in clinical trials. Effective concentrations of anisomycin, flavopiridol, and trichostatin A are in the submicromolar range. Tumor cell death can be prevented by epidermal growth factor (EGF), if added before flavopiridol or after anisomycin but not if applied between the additions of these agents, suggesting that flavopiridol interrupts an EGF-activated survival pathway and that anisomycin, besides triggering cell death, guards this pathway against the interference by flavopiridol. In contrast to EGF, dibutyryl-cAMP exerts protection that is flavopiridol-insensitive. For triggering cell death, anisomycin cannot be replaced by DNA- or mitotic spindle-targeted drugs in this system. The present findings, that a combination of transcriptional and signal transduction-targeted modulators sensitizes tumor cells syntergically to stress-mediated triggering of cell death and that ribotoxic stress is more efficient in this respect than genotoxic or spindle-targeted stress, bear important implications for the therapeutic exploitation of cellular stress responses. The stepwise sensitization and triggering of cell death in the present system allow separate analysis and manipulation of processes contributing to cellular death susceptibility and of the mechanism responsible for triggering cell death, thus providing the operational basis for further development of this therapeutic approach.

Introduction
Oncological therapies interfering with DNA replication or mitotic spindle formation can be viewed as stress-inductive treatments eliciting complex cellular responses including activation of protective functions like growth arrest, repair, adaptation, and survival mechanisms as well as of destructive pathways leading potentially to the execution of apoptotic cell death, which is the therapeutically favored outcome of the response (1, 2). An increasing body of literature demonstrates that complex molecular cell cycle checkpoint functions surveying DNA and mitotic spindle integrity (3–5) and a network of kinases (6) including the SAPKs (3, 4) (8–16) are involved in the coordination of these activities in accordance with the physiological state of the cell at the instant of treatment exposure. In normal cells, the engagement of cell cycle checkpoint functions by disturbances of DNA or spindle structure causes cell cycle arrest and activation of repair mechanisms and is believed to trigger an apoptotic response if the disturbance cannot be compensated otherwise (3–5). In contrast, as a consequence of their usually defective checkpoint and repair mechanisms, tumor cells are frequently neither efficient in arresting growth and repairing damage nor in triggering primary apoptotic responses to the treatment (3, 5). Instead, they perform aberrant DNA replications and/or mitoses leading to aneuploid karyotypes and chromosomal aberrations (3, 17–20). As a consequence of these secondary disturbances, part of the population usually dies (17–20), but another part escapes by adaptation and goes on to survive and to proliferate (3, 5, 19, 20), probably even with increased genomic instability and malignancy (3, 5, 20). This scenario is presumably, with individual variations, still the most common outcome of chemotherapy treatments presently applied in the clinics.

To prevent adaptation and escape during the lengthy development of secondary disturbances, it is proposed to increase the efficiency of the primary apoptotic response by a sensitization strategy, which concentrates in the first phase of the treatment, before triggering cell death, on increasing the relative efficiency of pathways leading from the trigger signal to the
execution of cell death. Furthermore, because effective triggering of a primary apoptotic response by DNA- or microtubule-targeted agents may be frequently impaired in tumor cells, we propose that alternative stress-inductive modalities should be considered and compared with the classic chemotherapeutic agents with respect to their potential to trigger a primary apoptotic response in appropriately sensitized tumor cells. Such a strategy could be fruitful not only by itself but also by complementing other approaches.

Recently, anisomycin has been shown to activate efficiently a cellular stress response involving the activation of SAPKs including JNKs (21) and p38/RK (22). Furthermore, it was demonstrated that this stress response originates from an interaction of anisomycin with ribosomal 28S RNA and that translationally active ribosomes are involved (23). This cellular response was therefore christened the ribotoxic stress response by Iordanov et al. (23, 24). Because this kind of stress signal primarily does not cause DNA damage or microtubular disarray and therefore presumably does not require respective cell cycle checkpoints for the effective activation of a primary apoptotic response, we investigated in the present work the suitability of anisomycin as a cell death trigger in human carcinoma cell populations, which are largely resistant to the induction of cell death by a panel of classic chemotherapeutic agents and by TNF (25). Because anisomycin by itself does not induce cell death in these cell lines either, we searched for sensitization modalities. For this purpose, we studied the effects of pretreatments with diverse agents, interfering with gene expression or signal transduction, on the susceptibility of these tumor cells to anisomycin-mediated triggering of cell death. In the present work, we describe efficient sensitization in vitro and in vivo by successive application of an inhibitor of histone deacetylation, such as TSA (26, 27) or butyrate, and of flavopiridol, a substance known to inhibit several CDKs (28, 29). TSA and butyrate have been shown to modulate transcription of genes involved in the control of cellular proliferation, death, and survival (30–36), and flavopiridol causes cytostatic and cytotoxic effects in several tumor cell lines (28, 37–39) and is presently being investigated in clinical trials as a potential anticancer agent (40). We furthermore report that, in comparison to classic chemotherapeutic agents, anisomycin is the superior trigger of tumor cell death in this system. First mechanistic data provide insight in a network of interacting cell death and cell survival pathways, which is appropriately tuned by the sensitization procedure on transcriptional and posttranscriptional levels to favor the fast and efficient triggering of apoptotic cell death by anisomycin.

Materials and Methods

Cell Culture. Stock cultures of the human lung (LT23) colon (HT29) and mammary (SKBR3) carcinoma cell lines were kept as described previously (25) in collagen-coated, 10-cm plastic dishes in a serum-free medium, consisting of a 1:1-mixture of DMEM and HAM’S F12 (Life Technologies, Inc.) supplemented with 25 μg/ml ampicillin and 50 μg/ml gentamicin and with additions of 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml epidermal growth factor, 0.1 nM triiodothyronine, 75 nM sodium selenite, 8 μM ethanalamine, 2.5 μg/ml oleic acid, and 0.6 mg/ml fatty acid-free albumin. For experiments, collagen-coated, 24-well plates (Greiner) were used, and 2 × 10^5 cells were seeded per well in 1 ml of the above medium without gentamicin. In case of experiments with TSA, the medium was complemented with 2% BSA, because TSA effects were found to be greatly diminished at the low albumin concentration of the basic medium. Cultures were routinely checked for Mycoplasma (Mycoplasma detection kit; Boehringer Mannheim) and as a precaution were subjected to a decontamination procedure with a combination of antibiotics (BM-Cyclin; Boehringer Mannheim) before frozen stocks were prepared.

Drug Application and Evaluation of Viable Cells. Twenty-eight h after seeding, the medium in 24-well plates was replaced by 1 ml/well of fresh medium containing sodium butyrate or TSA at the concentrations indicated in individual experiments. Sixteen h later, flavopiridol was added as concentrate in a volume of 100 μl of culture medium per well. The flavopiridol application time is the reference time and has been set at t = 0 h in all experiments. One hundred μl of appropriate anisomycin concentrations were added after flavopiridol at the times indicated individually. One to 3 h after anisomycin application, the medium was removed quantitatively and replaced by fresh culture medium without drugs. Surviving cell numbers were determined at t = 24 h, as described previously (41). Briefly, after removal of the medium, culture wells were exposed to 1.5 ml of a hypotonic detergent solution containing 0.25% Triton X-100 in 0.4 mM EDTA at pH 7.5. Under this condition, cell membranes of viable cells are lysed quantitatively, whereas nuclei are swollen and released as intact entities. After 10 min, the nuclear suspensions are fixed by addition of 0.5 ml of 10% formaldehyde, pH 7.0. Dead cells are pyknotic at this time of the experiment (t = 24 h) and do not release their nuclei. Total particle numbers are determined in this suspension by electronic counting (Coulter Counter ZM) and size distribution analysis (Coulter Channelizer C256). The percentage of nuclei from viable cells is determined by flow cytometry (Coulter EPICS XL) on the basis of their light-scattering signal, the intensity of which is about 1/100th to 1/1000th of the signal intensity generated by pyknotic cells (41).

Significance. Each experiment reported was repeated several times with qualitatively similar results. However, over longer time periods of weeks and months, basic sensitivity of the cell cultures fluctuated from experiment to experiment because of unknown reasons. Triplicates performed in individual experiments did not deviate by >10% from their mean value.

Animal Experiments. Athymic nude CD1 mice (Charles River), 12–16 weeks of age, received interscapular s.c. injections of 1.5 × 10^6 LT23 cells in a volume of 100 μl of culture medium/animal. Twelve days after inoculation, when pea-sized tumor nodules had formed, animals received 200 μl of TSA solution each into the tail vein at the following times: t = 16 h; t = −8 h; t = 0 h; and t = 3 h. One hundred μl of flavopiridol solution was given at t = 0 h. One hundred μl of anisomycin solution each was given at t = 3.10 and 3.25 h. The TSA solution contained 1 mg/ml TSA, 0.9% NaCl, 4.5% BSA, 2.5% DMSO, and 10 mM HEPES (pH 7.4). The flavopiridol solution contained 4.5 mM flavopiridol, 0.9% NaCl, 0.24% BSA, 9% DMSO, and 10 mM HEPES (pH 7.4). The anisomycin solution contained 3 mg/ml anisomycin, 0.9% NaCl, 0.24% BSA, 3% DMSO, and 100 mM HEPES (pH 7.4). Controls received injec-
sensitization to ribotoxic stress-induced apoptosis. At $t = 24$ h, animals were sacrificed. Tumors and organs were fixed in 3% formaldehyde for histological preparation and staining.

**Substances.** Flavopiridol was provided by Dr. Worland and the Drug Synthesis and Chemistry Branch at the National Cancer Institute (Bethesda, MD). TSA was initially a gift from Dr. Yoshida (Department of Agricultural Chemistry, University of Tokyo, Tokyo, Japan). Later it was purchased from WAKO Chemicals. Ag1478, SB203580, PD98058, and rapamycin were from Calbiochem; all other agents were from Sigma Chemical Co. if not stated otherwise.

**Results**

**Sensitization of Human Lung Adenocarcinoma Cells (LT23) by TSA and Flavopiridol to Anisomycin-triggered Cell Death.** In this report, we describe cell death-inductive synergisms in a human tumor model, which exist between inhibitors of histone deacetylation (TSA and butyrate), flavopiridol, and anisomycin. They are critically dependent on the schedule of drug application. The highest rates of cell death are induced if TSA is applied 12–24 h in advance of flavopiridol and anisomycin is added at least 3 h after flavopiridol. With these treatment modalities, cell death can be triggered by anisomycin in a highly synchronous manner, leading to cell losses of 90–100% within 1–3 h in LT23 cells (Fig. 1), which were shown previously to be resistant against TNF-$\alpha$ at dosages up to 70 ng/ml (25) and which proved during the present work to be resistant against the induction of cell death by 24-h exposures to 1.6 $\mu$g/ml epirubicin, 40 $\mu$M 5-fluorouracil, 2.4 $\mu$M etoposide, and 8 $\mu$g/ml cisplatin and to be moderately sensitive to 20 nM paclitaxel, which induced $\sim$50% cell loss within 24 h (data not shown). The omission of any one of the three agents leads to reduction of the extent of cell death by an order of magnitude (Fig. 1). Flavopiridol, in particular, as a single agent and the combination of TSA plus anisomycin do not induce significant death rates. Likewise, changes in the order of application lead to dramatic reductions of death rates (not shown). The effective concentrations are in the submicromolar range for all three agents (Fig. 2). In Fig. 3, two important dynamic features of the system are documented: (a) the extent of cell death triggered by anisomycin is highly dependent on the timing of the anisomycin exposure. Cell death rates increase significantly, if the interval between the additions of flavopiridol and anisomycin is increased from 0 h (Fig. 3, columns 2) to 3 h (Fig. 3, columns 4) at constant overall exposure time. It is to be emphasized that increasing death rates are observed here with decreasing anisomycin exposure times; (b) TSA-mediated sensitization is completely reversible, if TSA is withdrawn at least 4 h prior to the application of flavopiridol (Fig. 3, groups II–V). Furthermore, TSA-mediated sensitization can be suppressed by amanitin (Fig. 4) at concentrations of $\sim$0.3–1.0 $\mu$M, which are inhibitory for RNA polymerase II selectively (42). In contrast, sensitization by flavopiridol is reversible within 1 h of drug withdrawal and cannot be suppressed by amanitin (Fig. 5). The controls show that there is neither inhibitory nor synergistic cell death-inductive interaction between flavopiridol and amanitin. If anisomycin is replaced by 5-fluorouracil (up to 40 $\mu$M), etoposide (up to 2.4 $\mu$M), epirubicin (up to 0.8 $\mu$g/ml), cisplatin (up to 8 $\mu$g/ml), or Taxol (up to 40 nM), there are no significant cell death-inductive synergisms detectable, with the exception of epirubicin, which exerts a weak synergetic effect (Fig. 6).

**Suppression of Cell Death by EGF, Dibutyryl-cAMP, or Caspase-Inhibitor Z-Asp-DCB.** The LT23 cell line can adapt to grow permanently in the absence of EGF. During the adaptation period of about 2 weeks, growth rates are slightly reduced, and morphology changes from a more fibroblastic type in the presence of EGF to a more epithelial type in the absence of EGF. However, no crisis-like loss of major parts of the population is observed during adaptation. The adapted cell line is called LT/-E here. The reexposure to EGF causes effective protection of LT/-E cells against anisomycin-triggered cell death, if EGF is added shortly before flavopiridol or shortly after anisomycin application, but not if added in the time interval between the applications of flavopiridol and anisomycin (Fig. 7). However, cell death can be prevented effectively at all times during treatment up to 50 min past anisomycin addition by the caspase-inhibitor Z-Asp-DCB (Refs. 16 and 43; Fig. 7). If added.
before flavopiridol, the protective efficiency of EGF decreases rapidly, if EGF is added later than 20 min before flavopiridol (Fig. 8A). Protection by EGF is prevented completely by AG1478, a specific inhibitor of the EGF receptor tyrosine kinase (44), if added up to 2 min before EGF. If added after EGF, AG1478 effectiveness decreases with increasing delay to the EGF addition, becoming negligible between 15 and 30 min after adding EGF before flavopiridol or being significantly but not quantitatively reduced within 30 min of EGF addition after anisomycin (Fig. 8B). If EGF is added after anisomycin, protection by EGF is dependent on the time of anisomycin application (Fig. 9A) in the sense that the EGF protection maximum is shifted coherently with changing anisomycin application times, being detected always 25 min past anisomycin application, irrespective of the particular time of anisomycin exposure. Wortmannin, an inhibitor of PI 3K and related kinases (45), prevents EGF-mediated protection significantly, irrespective of the time of EGF addition (Fig. 9B), whereas PD98059, an inhibitor the MAPK/ERK cascade (46), at concentrations up to 20 µM does not diminish EGF-protective effects (data not shown). In addition to EGF, dibutyryl-cAMP was found to exert strong protective effects against cell death in the present system. However, in contrast to EGF, dibutyryl-cAMP causes protection rather independently of its application time point, even at times between the additions of flavopiridol and anisomycin, when EGF is without effect (Fig. 9C). Furthermore, protection by dibutyryl-cAMP cannot be suppressed by wortmannin (data not shown). SB203580, an inhibitor of p38-(IC50 between 0.1 µM and 1.0 µM) (47, 48) and at higher concentrations of various INK2-isozymes (IC50 10 µM and 100 µM (48) prevents cell death in LT23 cell cultures in a concentration-dependent manner if added before anisomycin (Fig. 9D).
effects were diarrhea and reduced spontaneous activity of the animals after the injection of anisomycin, lasting for 6–8 h. Twenty h after anisomycin application, when tumors and tissues were prepared for histological examination, an average weight loss of 5% was detected in treated animals, which otherwise seemed to have recovered from therapeutic side effects and were in excellent condition. Histological examinations of the host tissues including liver, kidney, lung, ileum, and colon at this time point revealed no obvious morphological changes. High rates of pyknoses, as observed in the tumor tissue, were not seen in any other organ or tissue.

Discussion

Anisomycin, SAPKs, and Cell Death. As an alternative to classic DNA- or microtubule-targeted oncological drugs, we have selected in the present work anisomycin as a stress-inducive therapeutic agent. This substance has been described as an efficient activator of SAPKs (21–23), a group of proline-directed kinases (7) belonging to the more extended network of MAPKs (6, 49), which is activated to varying degrees by DNA-targeted drugs (8, 12–14, 16, 50), microtubule-interfering agents (9, 11, 15), and a variety of other stressors (49, 51) such as oxidative toxicants, osmotic shock, heat shock, UV irradiation, proinflammatory cytokines, and growth factor withdrawal. SAPKs phosphorylate and activate several transcription factors (7, 48, 49, 51, 52) as well as targets directly involved in stress effector functions like the kinase MAPKAPK-2, which for example phosphorylates and activates the stress protein hsp27 (7, 51). In this way, SAPKs contribute to the intracellular development and propagation of stress responses via direct activation of effector functions as well as by activating new gene expression. In distinction to DNA- and microtubule-targeted chemotherapeutic drugs, anisomycin binds to the 28S RNA of the eukaryotic ribosome. SAPK activation by anisomycin has been shown to depend on translationally active ribosomes and can be prevented by prior inhibition of translational elongation (23). At the low concentrations and short exposure times used in the present work, anisomycin activates SAPKs efficiently in various cell lines (22, 23, 52). However, similar to the present work, the immediate induction of extensive cell death rates is usually not observed under these conditions (53), suggesting that at low anisomycin concentrations, protective pathways dominate over...
destructive ones in this stress response. In the present work, we have investigated the possibility of sensitizing human lung adenocarcinoma cells to the cell death-inductive action of anisomycin.

**Mechanistic Details.** The induction of tumor cell death by the sequential application of an inhibitor of histone deacetylation, flavopiridol, and anisomycin is a highly synergetic process, as flavopiridol by itself or the combination of TSA plus anisomycin are not effective. However, limited cell death is induced by combinations of flavopiridol with TSA or anisomycin (Fig. 1). Cell death in this system can be suppressed completely by the protease inhibitor Z-Asp-DCB (Refs. 16 and 43; Fig. 7), which inhibits a broad range of caspases, proteases typically activated during execution of apoptotic cell death (54, 55). This finding and the electron microscopic examination, which revealed, in dying LT23 cell populations, morphological characteristics of apoptosis [chromatin condensations, cytoplasmic blebbing, and apoptotic body formation (not shown; Ref. 56)], suggest that apoptotic cell death is induced in the present system. The concentration-dependent prevention of cell death by SB203580 indicates the involvement of p38/RK (47) and possibly JNK2 (48) in the triggering process activated by anisomycin. The activation of SAPKs has been correlated in other systems with the induction of apoptotic cell death (8, 9, 11, 16, 51, 53). However, a definitive mechanistic context has been demonstrated up to now only for a positive feedback loop between MEKK1, a kinase upstream of SAPKs, and caspase-3 (50). How and where SAPKs come into play is presently not known. The dramatic reductions of death rates upon withdrawal of TSA (Fig. 3) or flavopiridol (Fig. 5) within 4 or 1 h, respectively, document that the main effects of TSA and flavopiridol are reversible sensitizations with respect to the triggering of cell death by anisomycin. What is the mechanistic basis of these sensitizations? The amanitin sensitivity of the TSA effect (Fig. 4) and the insensitivity of the flavopiridol effect to amanitin (Fig. 5) suggest that transcriptional activation of gene expression is involved in TSA-mediated sensitization but not in flavopiridol-mediated sensitization. Thus, the sensitizing effect of inhibitors of histone deacetylation may be viewed in context with the concept that transcriptional competence of chromatin is controlled by dynamic histone acetylation and deacetylation (57, 58). Induction of and sensitization to cell death by histone deacetylase inhibitors has been reported in other systems also (36, 59–61). In this respect, the transcriptional regulation of genes involved in the control of cellular proliferation and death susceptibility has been described as a consequence of treatments with histone deacetylase inhibitors. Thus, down-regulation of the antiapoptotic regulators BCL2 (59) and BCL-xl (36) has been reported, as well as increased expression of the CDK-inhibitor p21/WAF1 (34) and a transient up-regulation of the stress proteins HSP27 and HSP70 (62, 63). Furthermore, complex modulation of diverse growth-regulatory genes, including c-fos and c-myc, was described in different systems (30–33). In contrast, flavopiridol is known as an inhibitor of the CDKs CDK1 (29), CDK2, and CDK4 (28). This agent has been shown to cause growth inhibition in diverse tumor cell lines (28, 39) and, if applied for 24 h, to exert cytotoxic effects
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Animal at times of EGF addition, susceptibility to inhibition by EGF. Sensitivity to EGF is maximal at times of maximum EGF-mediated protection on EGF application time relative to flavopiridol-addition (t = 0 h). B, prevention of EGF-mediated protection by an inhibitor of EGF-receptor tyrosine kinase (AG1478). LT23 cultures were exposed to the combination of 2 mM sodium butyrate, 750 nM flavopiridol, and 150 ng/ml anisomycin according to the schedule in Fig. 1, where TSA was replaced by butyrate in the present experiment. EGF was added to individual cultures at the indicated times. Drug exposure was terminated at t = 280 min. Survival was evaluated at t = 24 h. The cell count at t = −16 h (addition of TSA) was set at 100%. AG1478 (1 μM) was added at the indicated times. C, control without AG1478.

The most conspicuous feature of the mechanism, by which cell death is induced in the present system, is its biphasic susceptibility to inhibition by EGF. Sensitivity to EGF is maximal at times of EGF addition, ~30 min before flavopiridol and 30 min after anisomycin addition, respectively, and a complete lack of susceptibility to EGF-mediated protective effects is detected in the time interval between the additions of flavopiridol and anisomycin (Figs. 7 and 9A). The disappearance of cellular susceptibility to AG1478-mediated inhibition of the EGF-activated survival signal within 20 min after EGF addition (Fig. 8B) suggests that the EGF-generated survival signal is a pulse of ~20 min length. If flavopiridol is added after EGF within the EGF pulse time, the level of survival is continuously reduced with decreasing delay between EGF and flavopiridol additions (Fig. 8A). This suggests that flavopiridol interferes more or less instantaneously (within only seconds or few minutes) with the transmission of the EGF-generated survival sig-

nal. EGF-mediated protection can be suppressed partially by wortmannin (Fig. 9B), an inhibitor of PI3K but not by PD98059, an inhibitor of the MAPK/ERK pathway (46), or by rapamycin, an inhibitor of the p70-S6-kinase pathway (Ref. 64; not shown), suggesting that a PI3K-dependent pathway contributes to the transmittance of the survival signal. It has been shown in other systems that survival signals are propagated by a PI3K-dependent mechanism (65, 66) involving Akt/PKB-mediated phosphorylation of the proapoptotic BAD protein (67, 68), which in its unphosphorylated form prevents BCL-XL from exerting its anti-apoptotic function and in its phosphorylated form is sequestered by a 14-3-3 protein (68). Possible downstream effects of BCL-XL include its heterodimerization with the proapoptotic partner BAX (69) and/or more direct interactions with the caspase network (70–72). In contrast to BCL2, which we have not found to be expressed in LT23 cells, BCL-XL, BAD, and BAX can be detected in LT23 cell extracts by Western blotting.

Thus, a model consistent with these findings invokes EGF-mediated PKB activation and BAD phosphorylation as a possible survival pathway. In contrast to EGF, dibutyryl-cAMP experts protection against anisomycin-triggered cell death in the present system, not only if applied at the times of maximum EGF sensitivity but also if added at times between flavopiridol and anisomycin additions, when EGF is without protective effect. This means that cAMP either activates an alternative survival pathway, which is not accessible to flavopiridol interference, or feeds into the same pathway as EGF but, in contrast to EGF, after the point of flavopiridol interference. In further contrast to EGF, the protective effect of cAMP is not wortmannin sensitive (data not shown). In this context, it is interesting that cAMP has been shown to cause Akt/PKB activation in human embryonic kidney cells in a wortmannin-insensitive manner, involving phosphorylation of the same serine and threonine residues on PKB as PI3K-mediated activation (73). Thus, because PKB is not a direct substrate for PKA (the enzyme directly activated by cAMP), the convergence of PI3K-dependent and -independent pathways could occur at the level of the PKB-activating kinases phosphatidylinositol-dependent kinases 1 and 2 (66). Alternatively, BAD might be a direct substrate of PKA (68).

Interestingly, anisomycin does not only trigger efficiently apoptotic cell death but also restores cellular susceptibility to protection by EGF, which has been suppressed previously by flavopiridol (Fig. 7). Because flavopiridol is still present at the time of anisomycin exposure, this suggests that anisomycin makes the protective pathway activated by EGF inaccessible to the interference by flavopiridol or that it opens up a different, flavopiridol-insensitive way for the transmission of the EGF-generated survival signal. In this context, the significant reduction of death rates, which is observed, if anisomycin is added “too early” (Fig. 3, compare columns 2, 3, and 4), is interesting. If anisomycin is added together with flavopiridol, it could be speculated that the lowered death rates may be attributable to an early inhibition of the flavopiridol interference with survival pathways by an anisomycin-activated function, which comes

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late if anisomycin is added 3 h later. The efficiency of wortmannin to suppress the protective effects of EGF, even after anisomycin application (Fig. 9B), suggests that the survival pathway activated by EGF after anisomycin addition is the same one activated before flavopiridol application, which is also wortmannin sensitive. However, if EGF is added before flavopiridol, wortmannin can only suppress part of the EGF-dependent survival (Fig. 9B), suggesting that a wortmannin-insensitive pathway contributes to the propagation of this survival signal as well. A candidate for this function might be a RAF-1-dependent survival pathway, which was found to be activated in another system (74).

Working Model. In Fig. 11, the present findings and some open questions are summarized in the framework of a speculative model invoking the survival and death pathways discussed above as a mechanistic basis. In this model, it is assumed that cell death is effected by caspases and cell survival by BCLXL-dependent inhibition of caspases, which is activated by survival effector kinases (RAF-1, PKA, and PKB), phosphorylating BAD and thereby releasing BCLXL. Flavopiridol causes complete blockade of an EGF-activated survival mechanism, part of which is wortmannin sensitive. Whether flavopiridol exerts this blockade by inhibition of CDKs or by a different mechanism is not clear at present. In addition, flavopiridol may increase the efficiency of the apoptotic trigger mechanism, e.g., by releasing SAPK-mediated caspase activation from hypothetical CDK-dependent inhibitory phosphorylations. cAMP prevents cell death in a wortmannin- and flavopiridol-insensitive manner. Anisomycin activates SAPKs. Because anisomycin does not trigger only cell death but also reopens a wortmannin-sensitive survival pathway to the activation by EGF, which was shut down by flavopiridol before, it is assumed that SAPKs are involved in caspase activation as well as in reactivation of the PI3K-dependent survival pathway. In view of multiple SAPK species (7) potentially activated by anisomycin, the kinases involved in triggering cell death need not be the same as the ones favoring survival. These are open questions. TSA may modulate transcriptionally several of the components included in this scheme and even other ones contributing in different ways to cellular death susceptibility. However, one product shown to be down-regulated by butyrate in another system is BCLXL (36).

A New Therapeutic Concept. With classic chemotherapeutic approaches, cell death-inductive stress is frequently present experiments. Survival was evaluated at t = 24 h. The cell count at t = 16 h (addition of TSA) was set at 100%. A. anisomycin was added to individual cultures as indicated at t = 90 min, t = 180 min, or t = 275 min. EGF was added at the indicated times to individual cultures. Drug exposure was terminated 70 min after anisomycin application, respectively. B, EGF was added to individual cultures either before flavopiridol at t = 30 min (left) or after anisomycin at t = 210 min (right). Wortmannin (400 nM) was added at the indicated times. Controls without EGF (C1) or with EGF (C2) were not treated with wortmannin. Drug exposure was terminated at t = 250 min. C, individual cultures received dibutyryl-cAMP (0.2 mM) at the indicated times. Columns C, control without dibutyryl-cAMP. Drug exposure was terminated at t = 270 min. D, SB203580 was added to individual cultures 5 min before anisomycin at the indicated concentrations. Drug exposure was terminated at t = 250 min.
**Fig. 10** Systemic induction of pyknoses in nude mouse transplants of LT23 carcinoma cells. Shown are a histological section of an untreated LT23 transplant (A) and two sections from different regions of a LT23 transplant treated with TSA, flavopiridol, and anisomycin as described in “Materials and Methods.”
not generated instantaneously as a direct consequence of drug application but rather accumulates as a consequence of secondary disturbances like chromosomal aberrations (3, 5, 17–20). The slow development of cell death-inductive stress under these conditions favors cellular adaptation, resistance, and escape mechanisms (3, 5, 20). The data reported in the present work document the efficient sensitization of initially resistant human lung adenocarcinoma cells to the activation of a primary apo-ptotic response by anisomycin, a ribotoxic stressor (23), avoiding the engagement of DNA or microtubule checkpoints. Sensitization is effected by sequential application of an inhibitor of histone deacetylation and flavopiridol, a protein kinase inhibitor, shown previously to inhibit CDKs. For inducing maximum death rates, all three agents have to be present until the end of the entire treatment, because the sensitizing effects of TSA and of flavopiridol are reversible within 4 and 1 h, respectively (Figs. 3 and 5). These results have been paralleled in experiments with the human colon carcinoma cell line HT29 and the human breast carcinoma cell line SKBR3, which were less sensitive than the LT23 lung carcinoma but otherwise responded to the treatment equally with high primary apoptotic death rates (data not shown). This finding suggests that the proposed strategy may be of general value for the induction of primary apoptotic responses in human carcinomas. First systemic (i.v.) application trials in vivo have yielded high rates of pyknoses in nude mouse transplants of the presently investigated lung adenocarcinoma LT23 within 6 to 18 h of the anisomycin application in the absence of similar developments in the normal host tissues. These findings document that the therapy is tumor cell specific with respect to the induction of a primary apoptotic response and that the therapy penetrates into the tumor tissue in vivo, causing cell death with similar kinetics as in vitro. At present, the mechanistic basis for this selective induction of tumor cell pyknoses is not known. The superior efficiency of anisomycin as an apoptotic trigger, in comparison with DNA- or microtubule-targeted drugs, suggests that the exploitation of the ribotoxic stress response for therapeutic purposes might be a fruitful line to follow, especially in view of the nonmutagenic nature of the agents used in this approach. Although we do not understand at present the molecular mechanisms responsible for sensitization by TSA and flavopiridol or for triggering by anisomycin, our results provide first insights into a dynamic interplay of cell death and cell survival pathways, which can be controlled by transcriptional and signal transduction-targeted modulators. These insights were possible on the basis of the highly structured organization of the present strategy allowing stepwise sensitization by distinct interference with different levels of cellular organization and separate triggering of the apoptotic response. This feature will make it easier to unravel mechanisms and molecular entities contributing to the various aspects of the overall process. It will also be the operational basis for further development of this kind of therapeutic approach, endowing it with a high potential for discrete modifications as a consequence of new mechanistic insights or in response to specific therapeutic requirements of different categories of tumors or with respect to potential side effects. The failure of genotoxic drugs to replace anisomycin as an efficient trigger agent in the present system (Fig. 6) might be explained by one or several of the following reasons: (a) enhancement by TSA and flavopiridol might be specific for anisomycin-triggered apoptotic signaling pathways; (b) the short exposure time of 1–2 h, which suffices for anisomycin, might not be sufficient for genotoxic agents to trigger the apoptotic reaction; and (c) specific or general drug resistance mechanisms not encompassing anisomycin, flavopiridol, or TSA might contribute to this difference as well.
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