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Perspectives in Cancer Research

ATP Depletion + Pyrimidine Depletion Can Markedly Enhance Cancer Therapy: Fresh Insight for a New Approach[1]

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Introduction

In the 1980s, necrosis was considered the mode of cell death induced by DNA-damaging anticancer agents because of the activity of PARP.[3] PARP is activated by the DNA strand breaks caused by anticancer agents and cleaves the glycolytic coenzyme, NAD^+, leading to formation of poly(ADP-ribose) moieties. The ensuing depletion of NAD^+ inhibits glycolytic generation of ATP with consequent ATP depletion, eventuating in necrotic cell death (Fig. 1).

Heterogeneous neoplastic cell populations likely contain cancer cells of variable sensitivity to the anticancer agents. Less sensitive cells would not receive enough damage to reduce ATP to low levels sufficient to cause necrotic death. We hypothesized that biochemical modulation to further depress ATP to lower lethal-inducing levels would kill these sublethally injured cells, augment tumor regressions, and perhaps even yield some cures.

The ATP-Depleting and Pyrimidine-depleting Agents

Biochemical modulation is the manipulation of intracellular metabolic pathways by agents to produce selective enhancement of antitumor effects by the anticancer agent (1). Because damage to the glycolytic generation of ATP in cancer cells was shown to occur after the administration of DNA-damaging anticancer agents (2–7), 6-AN, an NAD antagonist, known to inhibit glycolytic production of ATP (8–13), was administered with anticancer agents to further deplete intracellular ATP.

MMPR, known to inhibit *de novo* purine biosynthesis (14, 15) and thereby limit adenine supplies for ATP production, was also concomitantly administered. In high dosage, MMPR also decreases pyrimidine ribonucleotide concentrations *in vitro* (16). Because a *de novo* pyrimidine synthesis inhibitor, PALA, as a single agent in low nontoxic dosage can selectively lower pyrimidine nucleotide levels in tumors (17), low-dose PALA was added to MMPR therapy to further lower the reduction of pyrimidine synthesis by MMPR. The three agents PALA, MMPR, and 6-AN were evaluated alone, in various double combinations, and as a triple combination against advanced breast tumors in mice. Pooled experiments (18, 19) demonstrated that neither the maximum tolerated dose of MMPR alone, nor 6-AN alone, nor the double combination of PALA + 6-AN produced cell kill. There were no partial regressions of tumors (PR, ≥50% tumor shrinkage in the volume of the initially measured tumor). However, tumor growth was inhibited in these groups as compared with saline controls.

Cell cycle events (*i.e.*, proliferation) require a minimal ATP content to undergo proliferation. If ATP depletion is reduced to levels >15% of normal but is below the minimal level necessary for cell division, only proliferation arrest (*i.e.*, tumor growth inhibition) and not cell death (*i.e.*, tumor regression) will ensue (20, 21). Table 1 records that MMPR alone (group 1) and 6-AN alone (group 2) depress tumor ATP levels 48 h after treatment to 34 and 69%, respectively, compared with saline-treated control tumors. These are ATP levels compatible with the tumor growth inhibitions produced by MMPR alone and 6-AN alone in the above-pooled published experiments (18, 19). PALA does not effect ATP depletion and in the low dosage that was administered reduces pyrimidine biosynthesis but does not have anticancer activity (17). Hence, the above-noted combination of low dose PALA + 6-AN only inhibited tumor growth attributable to the 6-AN, which alone only reduced ATP to 69% of normal (48 h, group 1; Table 1).

In contrast, the double combination of MMPR (a strong ATP depleter, 34% of normal, 48 h, group 1; Table 1) plus PALA (which is devoid of an ATP-depleting effect) produced a very few partial tumor regressions, 7% PR (18, 19). The MMPR-induced depletion of ATP to 34% is an average; hence, a few individual tumors likely have an ATP level ≤15% of normal, a level shown to be insufficient to sustain cell viability (20, 21), and particularly in the presence of the severe pyrimidine depletion produced by the double combination of PALA + MMPR, as is explained below.

Please note that the murine tumors in these experiments are first-passage s.c. transplants from a tumor brei made by mixing the cancer cells of three or four single, spontaneous, autochthonous breast tumors, the CD_8F_1, tumor model included previously in the National Cancer Drug Screening Program (23–25). All spontaneous tumors, whether human or murine, have a heterogeneous neoplastic cell population. Because each experiment consists of a brei composed of several different spontaneous tumors, the neoplastic cell composition is somewhat different form experiment to experiment, resulting in some quantitative differences between experiments. However, each experiment has its own control, and the results are quantitatively relevant within individual experiments, as are trends among experiments.

In this series of three pooled published experiments, the double combination of MMPR + 6-AN produced an objective response rate of 17% PR (18, 19). This therapeutic result is compatible with the MMPR + 6-AN-induced cell killing average ATP level of 15% of normal (Table 1; group 3, 48 h). Note that the low ATP level of 15% induced by MMPR + 6-AN is, as expected, unchanged (still 15%) by the addition of PALA to MMPR + 6-AN (group 4; Table 1). However, in the presence of this severe limitation to ATP availability (15% of normal), the triple drug combination of MMPR + 6-AN + PALA produced a PR rate of 61% (18, 19). The severely depleted ATP levels likely inhibit the salvage pathway formation of pyrimidine di- and

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[3] The abbreviations used are: PARP, poly(ADP-ribose) polymerase; 6-AN, 6-aminonicotinamide; MMPR, 6-methylmercaptopurine riboside; MMPR-P, MMPR-phosphate; PALA, *N*-(phosphonacetyl)-L-aspartic acid; PR, partial regression; MAP, MMPR + 6-AN + PALA; FU, 5-fluorouracil; Adr, Adriamycin; MTAP, methylthioadenosine phosphorylase; MPT, mitochondrial permeability transition; IAPS, inhibitor(s) of apoptosis.

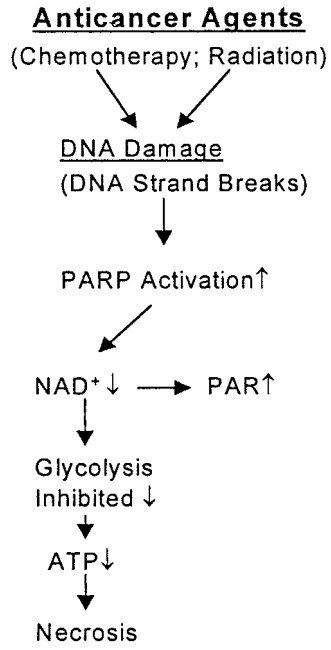

Fig. 1. DNA strand breaks activate PARP, which cleaves NAD into PAR moieties. The result is a marked decrease in NAD^+ with a consequent fall in ATP until finally there is insufficient ATP to sustain survival of the cell, and cell death by necrosis occurs.

triphosphates at the kinase step. Pyrimidine nucleotides serve essential functions in nucleic acid metabolism and sugar nucleotide formation for glycosylation of proteins and lipids. It is not surprising that severe inhibition of pyrimidine biosynthesis (because of PALA + high-dose MMPR), in the presence of severe ATP depletion (because of MMPR + 6-AN), enhances tumor regressions over MMPR + 6-AN (18, 19). The UTP pools in the *in vivo* MAP-treated tumors were sharply reduced to 14% of normal (18). It is the severe lowering of ATP levels (15% of normal) that is the key ingredient that allows the severe pyrimidine depletion to appreciably augment the anticancer activity of MAP (a 61% PR) over that of MMPR + 6-AN (a 17% PR).

Although MMPR + 6-AN causes the all-important ATP depletion to cancer cell-killing levels of ≤15% of normal (20, 21), another reason for including PALA with MMPR + 6-AN (*i.e.,* MAP) is pertinent to the administration of DNA-damaging anticancer agents. The induction of apoptosis by the anticancer agents causes mitochondrial damage in sublethally injured cancer cells. Pyrimidine *de novo* synthesis is functionally linked to the respiratory chain in the inner mitochondrial membrane by mitochondrial-bound dihydrooratate dehydrogenase, the fourth enzyme of *de novo* pyrimidine synthesis. Thus, PALA (+ high-dose MMPR) should further lower the reduction of pyrimidine levels attributable to the mitochondrial damage effected by an anticancer agent-induced apoptotic biochemical cascade in surviving but sublethally injured cells. It has been shown previously that cells that had been completely depleted of mitochondria become pyrimidine auxotrophs because of the deficiency of the respiratory chain-dependent dihydrooratate dehydrogenase (26). A minimal level of pyrimidine nucleotides is essential to sustain cell life. MAP severely reduces both ATP and pyrimidine levels in cancer cells. In cancer cells sublethally injured by anticancer agents, ATP and pyrimidine levels are depleted by the mitochondrial damage induced by the apoptotic biochemical cascade initiated by the anticancer agent. Anticancer agents produce a tumor regression rate by directly killing many cancer cells by either necrosis or apoptosis, but they also effect sublethal injury to less sensitive cancer cells from which they will recover. MAP targets their sublethally injured cancer cells before they can recover, further decreasing their ATP and pyrimidine levels, killing these cells, and thereby markedly enhancing tumor regressions. It is the anticancer agents that preferentially reduce ATP and pyrimidines, two metabolites that are essential for cell viability, to low levels in sublethally injured cancer cells, thereby creating a therapeutic opportunity for biochemical modulation (*e.g.,* MAP) to further reduce them to lower levels insufficient to sustain the recovery of these injured cancer cells.

The central importance of severe ATP depletion to the tumor regressions (*i.e.,* cancer cell deaths) produced by MAP is illustrated in our *in vivo* experiments published previously (27) investigating the prolonged retention (4 days) of intracellular MMPR-P after MAP administration to mice bearing advanced tumors. MMPR is phosphorylated by adenosine kinase to MMPR-P, which inhibits *de novo* purine synthesis at the level of amidophosphoribosyl transferase, and this inhibition causes ATP depletion. The MMPR depletion of ATP is driven by prolonged MMPR-P levels over an extended period (4–5 days) because of continuous resynthesis of MMPR-P by adenosine kinase. After MAP administration, tumor ATP measurements (% of control) on days 2, 3, 4, and 5 averaged 52, 38, 35, and 50%, respectively, and MMPR-P was retained in the tumors at a high level over this prolonged period. The average ATP measurements of 38 and 35% likely include cell-killing ATP values ≤15% of normal because three partial tumor regressions were produced among 10 advanced tumor-bearing mice. Another group of 10 mice bearing the same transplants of advanced tumors received the same MAP treatment, followed 6 h later with iodotubercidin, an inhibitor of adenosine kinase, to allow an initial period of synthesis of MMPR-P prior to inhibition of adenosine kinase by iodotubercidin. However, this treatment prevented both the prolonged accumulation of MMPR-P and strong ATP depletion, producing tumor ATP values (% of control) of only 56, 53, 74, and 88% on days 2, 3, 4, and 5. In the presence of such poor ATP depletion, there were no partial tumor regressions.

The data (27) demonstrate that severe ATP depletion is necessary and central to MAP-induced tumor regression. Pyrimidine depletion (*i.e.,* PALA) makes a substantial contribution to achieving still more cancer cell deaths (*i.e.,* greater tumor regressions) only in the presence of severe ATP depletion. The biochemical damage done to sublethally injured cancer cells by anticancer agents renders these cells vulnerable to cell death by severe ATP-pyrimidine depletion.

ATP depletion clearly occurs, even without the ATP-depleting contribution of an apoptosis-inducing DNA-damaging anticancer agent (Fig. 1). The combination of only MMPR + 6-AN has been shown to effect severe lowering of tumor ATP levels in tumor-bearing animals. Specifically, the tumors of mice treated with MMPR + 6-AN (Group 3; Table 1) show a depletion to 15% of normal 48 h after administration (28).

MAP + Radiotherapy

The MAP regimen, when combined with radiation, produced cures for the first time in the murine advanced spontaneous breast tumor system, demonstrating the potential for this new therapeutic approach to convert merely palliative (*i.e.,* temporary tumor remission) treatment to curative therapy (29). Cures are claimed because the advanced murine tumors (treated when the tumor-bearing mice were 3 months old with only three intermittent courses of MAP + radiation every 10–11 days ending at day 21) underwent complete tumor regressions, which continued in 25% of the mice for >1 year (380 days). In contrast, no complete regressions were obtained with MAP alone, and only one short-lived complete tumor regression was obtained in animals treated with radiation alone.

Table 1 *Effect of MMPR + 6-AN + PALA on tumor ATP pools in CD_8F_1 mice*[a]

Group	Treatment	6 HR[b] μg ATP/mg ± SE	% saline control	24 HR[a] μg ATP/mg ± SE	% saline control	48 HR[a] μg ATP/mg ± SE	% saline control	72 HR[a] μg/ATP mg ± SE	% saline control
1	$MMPR_{150}$	3.3[d] ± 0.32	47	3.3[b] ± 0.96	47	1.6[b] ± 0.59	34	9.0[a] ± 2.4	127
2	$6\text{-}AN_{10}$	5.5[b] ± 0.21	77	5.3[b] ± 0.136	75	4.9[b] ± 0.16	69	12.0[d] ± 3.9	169
3	$MMPR_{150}$ + $6\text{-}AN_{10}$	4.1[b] ± 0.49	58	2.2[b] ± 0.08	31	1.1[b] ± 0.36	15	16.0[d] ± 3.6	225
4	MAP	3.9[b] ± 0.11	55	2.3[b] ± 0.31	32	1.1[b] ± 0.14	15	8.1[d] ± 0.7	114

[a] Subscript = mg/kg body weight; i.p. injections; first passage tumor transplants of CD_8F_1 spontaneous breast tumors.
[b] Statistical comparison to group 1 (saline control); significant = $P \leq 0.05$.
[c] Mean ± SE of 10 tumors/group (11 experiments).
[d] Mean ± SE of 6 tumors/group (4 experiments).

Summary of Preclinical Therapeutic Results with MAP + Cancer Chemotherapy

MAP plus each of eight mechanistically different anticancer drugs were administered to advanced tumor-bearing mice with a variety of tumor types (murine breast cancers, colon tumors, leukemia, and human breast cancer xenografts). The biochemical modulatory effort with MAP dramatically enhanced treatment of these tumors with agents that included doxorubicin, paclitaxel, cisplatin, 5-fluorouracil, phenylalanine mustard, cyclophosphamide, mitomycin C, and etoposide (29–36). The overall antitumor results with a variety of anticancer agents demonstrated safe and impressive significant augmentation of tumor regression, including complete regressions, and even some (25%) cures (29).

The addition of MAP to combination chemotherapy with two anticancer agents (FU + Adr) was safe, without need for dose reduction, and yielded enhanced antitumor activity, including complete regressions not achieved previously (32). The results encourage the prospect of the safe addition of MAP to a large number of anticancer agents in combination with the likelihood of even greater anticancer results (*e.g.*, after increased complete regressions comes cures).

Preclinical MAP Toxicity

MAP can cause body weight loss in mice. However, this weight loss is not accompanied by diarrhea or by histopathological changes in organs (such as the intestine). A severe decrease in eating and drinking for 3–4 days after each of the three courses of intermittent chemotherapy was noted. Treatment-conditioned weight loss because of failure to eat or drink is not unusual for animals receiving intensive chemotherapy. Importantly, weight loss, which can indeed cause inhibition of tumor growth, does not produce tumor regression. The therapeutic activity measured in all of our studies used the stringent clinical criterion of tumor regression (*i.e.*, 50% or greater decrease in tumor size). We have done separate experiments (unpublished) demonstrating that weight loss does not cause tumor regression. This fact is also clearly apparent in some of our published studies with ATP-depleting therapy. For example, in a pooled series of six experiments, two groups had similar weight loss (-17 and −19%), but one group had 60% tumor regressions and the other had only 2% tumor regressions. Also, in that same series of experiments, two other groups had identical weight loss (−25%) but different tumor regression rates (60% *versus* 79%) that were statistically significant. Weight loss would not be a problem in patients who, unlike animals, can be persuaded to drink and eat or can be supported i.v.

ATP Depletion in Tumors with MTAP Deficiency

MTAP, an enzyme involved in purine metabolism, is present in normal tissues but frequently is deleted (deficient) in leukemias, brain tumors, non-small cell lung cancers, breast cancers, melanomas, pancreatic cancers, and sarcomas (37–42).[4] Methylthioadenosine is produced during polyamine synthesis and cleaved to adenine and 5-methylthioribose-1-phosphate by MTAP. The adenine is reconverted to AMP and then to ATP. The deletion of the *MTAP* gene in many tumors results in the inability of these cancer cells to salvage adenine; the ATP pools in these cells must be depleted. L-Alanosine, a potent inhibitor of *de novo* AMP synthesis has demonstrated selective anticancer activity *in vitro* in MTAP-negative cell lines as compared with MTAP- positive cell lines (42).

An examination of MTAP expression in 10 human soft tissue sarcoma cell lines found MTAP not detectable in 3 of the 10 cell lines. These three cell lines were >10-fold more sensitive to L-alanosine than the cell lines containing MTAP. The addition of the *de novo* purine synthesis inhibitor, MMPR, further enhanced the sensitivity of the cells lacking MTAP activity to L-alanosine. These results provide the basis of selective therapy using L-alanosine + MMPR to treat patients with soft tissue sarcomas and are another example of the therapeutic utility of the ATP-depleting strategy. *In vivo* studies of L-alanosine + MMPR, as well as the addition of 6-AN, are being evaluated (43).

Recognition of Apoptosis as the Mechanism of Cancer Cell Death by Effective Anticancer Therapy

By the 1990s, apoptosis (44), a physiological mechanism for controlled cell deletion that is an energy-dependent, inherent gene-directed program of cell death, sometimes referred to as cell suicide and programmed cell death, was considered the cause of anticancer agent-induced cancer cell death (45, 46). Apoptosis and necrosis are considered separate entities, not only morphologically but mechanistically. It is generally believed that clinically effective anticancer agents, despite having different primary biochemical targets, *e.g.*, DNA damage by topoisomerase inhibitors, microtubule damage by paclitaxel and Taxotere, Fas antibody ligand binding to a Fas cell membrane surface receptor, and radiation damage to cell membrane sphingomyelin, all ultimately kill by inducing the biochemical cascades of apoptosis and necrosis (45, 46).

The sequential biochemical steps of apoptosis are schematically outlined in Fig. 2. Mitochondria play a central role in apoptosis (47). Anticancer agent-induced DNA damage effects a fall in the MPT (47, 48). The MPT fall releases apoptosis-inducing stimuli, reactive oxygen species, Bax (a proapoptotic protein), and Ca^{2+} overload (47, 48), are all factors that facilitate rupture of mitochondria. Mitochondrial rupture releases cytochrome *c* and procaspase-9 to join with cytosolic Apaf-1 and ATP in an apoptosome, leading to the activation of caspase-9 (49, 50). Activated caspase-9 then leads to other caspase-caspase interactions that activate caspase-3, caspase-6, and caspase-7 and the consequent cleavage of key substrates by the activated caspases (51, 52). Caspases, cysteine aspartate proteases, are active in proteolysis, and the result is the dismantling of the cell with the

[4] Unpublished results.

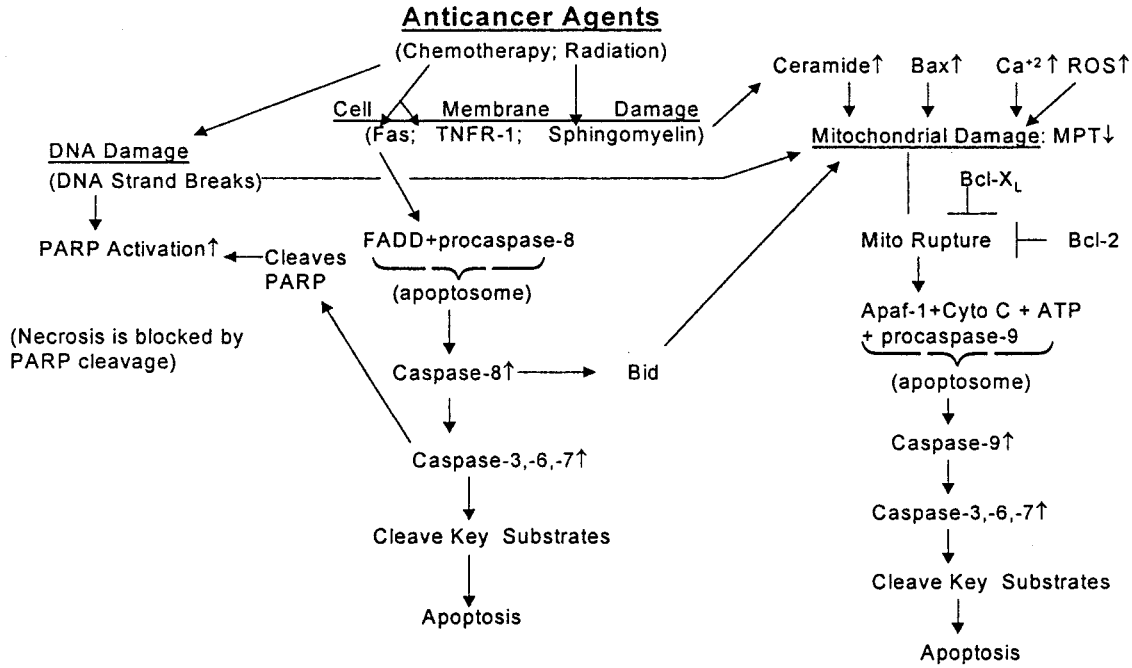

Fig. 2. Schematic outline of sequential biochemical pathways to apoptosis induced by anticancer agents. *Fas*, cell surface membrane receptor; *TNFR-1*, tumor necrosis factor receptor-1; ROS, reactive oxygen species; *Cyto c*, cytochrome *c*; *Mito*, mitochondrial.

morphology of apoptosis (51, 52). Radiation injury to cell membrane sphingomyelin activates the sphingomyelin signaling system to induce apoptosis (53). Ceramide is the second messenger of this pathway and is generated by hydrolysis of plasma membrane sphingomyelin through the action of either a neutral acidic sphingomyelinase (53) or by *de novo* synthesis via the enzyme ceramide synthase (54). Bcl-2 and Bcl-x_L are antiapoptotic proteins that protect mitochondria from loss of mitochondrial membrane potential (55, 56). The release of caspase-8 (48) by Fas activation leads to direct activation of the caspase system to cleave key substrates, dismantling the cell by apoptosis (51). Caspase-8 can also activate the proapoptotic protein, Bid, that can lead to mitochondrial rupture with activation of the mitochondrial-induced caspase/apoptotic death response system (48, 57). Caspase-3 cleaves PARP, halting the pathway to ATP depletion-induced necrosis via PARP-induced NAD^+ depletion (58, 59). Thus, the destruction of PARP activity permits caspase activity to complete apoptosis before PARP-induced ATP depletion causes necrotic cell death. It also should be noted that microtubule drugs induce apoptosis, and that there is evidence that interactions between the mitochondria and the cytoskeleton permit microtubule-active drugs to suppress the closure of the permeability transition pore in tumor mitochondria (60).

Controversy over ATP Depletion and Apoptosis

It is 28 years since Kerr *et al.* (in 1972; Ref. 44) first outlined the morphological criteria that distinguished cell death by apoptosis from necrosis. Many years passed before apoptosis became a biological subject of widespread and great scientific interest. Elucidation of its biochemical mechanism essentially began in the early 1990s. Thus, in the late 1980s, the preclinical therapeutic findings with the MAP program based on enhancing cell death by modulating ATP depletion was still compatible with the existing knowledge that necrosis was the mode of anticancer agent-induced cancer cell death.

However, by the early 1990s most clinically effective anticancer agents were considered to kill cancer cells by apoptosis (45, 46), and the presence of ATP was considered necessary for apoptosis (22, 61–63). For example, ATP is necessary for conversion of procaspase-9 to activated caspase-9 (50). Thus, the remarkable antitumor effects of MAP attributed to MAP-induced ATP depletion was questioned.

New Facts and New Insights into the ATP-Depletion/Necrosis/Apoptosis Paradox

Although clinically effective anticancer agents frequently kill cancer cells by activation of the biochemical cascade of apoptosis (45, 46), the same anticancer agents can induce cancer cell death by necrosis (56, 64, 65). Moreover, these two modes of cell death can occur in different cells simultaneously in tumors and cell cultures exposed to the same agent (56, 64, 65). The particular mode of cell death induced after drug treatment is dependent on the drug, its concentration, and the particular cell line (65). Because ATP depletion is the cause of necrosis, whereas ATP is necessary for apoptosis, it is noteworthy that necrotic and apoptotic cell death occur in the same tumor (but in different cells) after anticancer treatment. One reason is that different drug concentrations reach different cancer cells; low concentrations induce apoptosis, and higher concentrations cause necrotic cell death (65). However, this is not the only reason.

Because activated caspases execute apoptosis, it is noteworthy that the apoptotic mode of cell death can be prevented by an inhibitor of caspases (*e.g.*, Z-VAD-fmk), but instead of cell survival there is a shift to the necrotic mode of cell death (66–72). The reason is that severe ATP depletion, causative of necrosis, is brought about both by the fall in MPT (52), effecting a cessation of mitochondrial oxidative phosphorylation that generates ATP, as well as the block of caspase-3 by Z-VAD-fmk preventing caspase-3 cleavage of PARP, the result being continued PARP activity leading to NAD^+ depletion and consequent ATP depletion.

It is important to note that there are genetic deletions of caspases (73, 74), and there are endogenous IAP, *i.e.*, caspase inhibitors (75). Because apoptosis is governed by activated caspases, genetic loss of caspases or block by IAP of caspase activity, prevents apoptosis. In

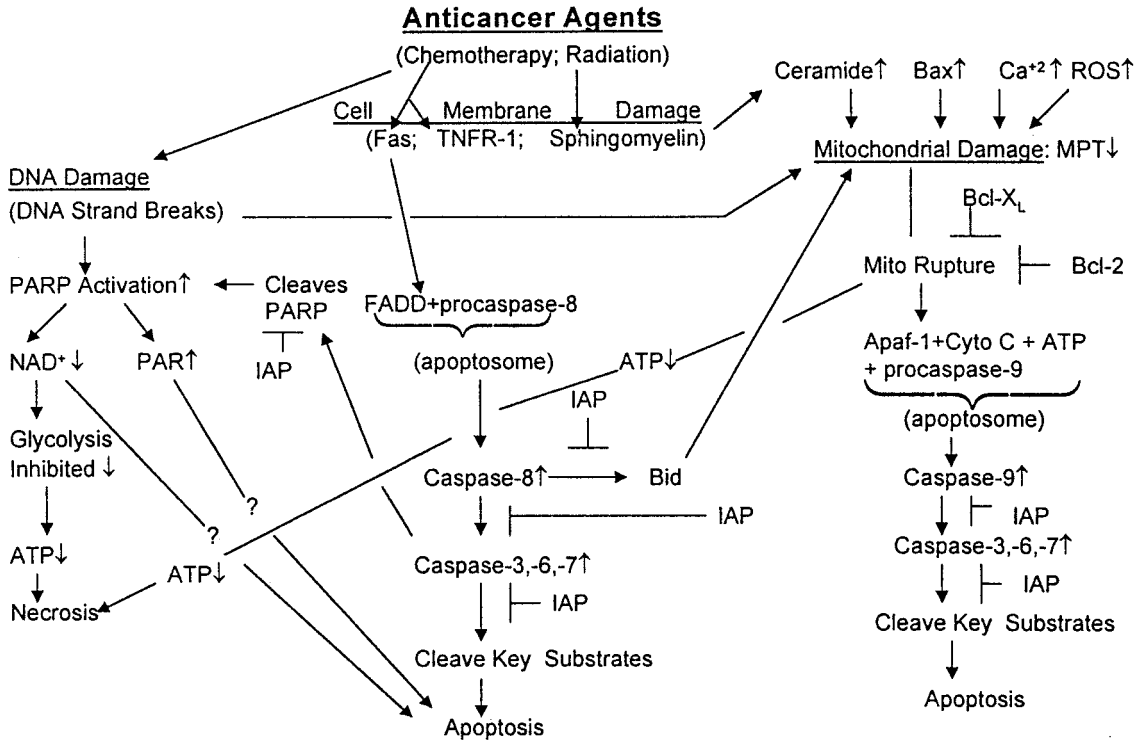

Fig. 3. Schematic outline of necrotic and apoptotic pathways with endogenous inhibitors of apoptosis, IAP, *i.e.,* inhibitors of caspases (75). If PARP cleavage is prevented, the continued activity of PARP leads to enhancement of both necrosis and apoptosis (58, 76). *?*, the possible relevance of NAD^+ levels and PAR, poly(ADP-ribose) polymers, to the enhanced apoptosis is not known.

Fig. 3, caspase inhibition by IAPs plus continued activity by PARP (note in Fig. 3, PARP cleavage by caspase-3 is blocked by an IAP), plus the ATP depletion from the loss of electron transport in ruptured mitochondria, drive the cell to necrosis largely because of continuation of PARP-induced ATP depletion (52, 76). It is believed that the purpose of PARP cleavage is to prevent induction of necrosis during apoptosis and ensure appropriate execution of caspase-mediated apoptosis (76). Failure of PARP cleavage (*e.g.,* by IAP-blocked caspases) would be expected to lead to the increased induction of necrosis but, surprisingly, is also reported to enhance apoptosis (58, 76). The question marks in Fig. 3 indicate that whether this enhancement is influenced by the continued PARP synthesis of PAR or by a relationship to the NAD^+ level is not understood (58, 76).

In brief, intracellular ATP levels may determine whether anticancer agent-induced cell death fate is by necrosis or apoptosis (77, 78). The activation and action of caspases, before ATP depletion can fall to levels causing cell death by necrosis, allows for caspase-executed apoptosis, and the availability of caspases *versus* IAP can dictate the propensity of cells to die from apoptosis *versus* necrosis. There are many reports of inhibition of caspase activity not conferring a survival advantage because the result is a shift from apoptotic cell death to necrotic cell death (22, 52, 56, 63, 64, 66–72, 77, 78).

A recent review article on mitochondria and apoptosis (52) states that, "The emergent view is that once cytochrome *c* is released . . . (by mitochondrial rupture) . . . , this commits the cells to die by either an apoptotic mechanism involving Apaf-1-mediated caspase activation or a slower necrotic process due to collapse of electron transport, which occurs when Cyto C is depleted from mitochondria resulting in a variety of deleterious sequelae including generation of oxygen free radicals and decreased production of ATP."

All of the above observations reveal that, rather than functional opposition between the two types of cell death, necrosis and apoptosis, there is a functional cooperativity (Fig. 3). The therapeutic implications are that a heterogeneous neoplastic cell population of a tumor likely includes cells with IAP, gene deletions of certain caspases, and lower levels of Bax. These cancer cells are likely to be of lesser sensitivity to an anticancer agent and escape death because they do not receive enough damage to reduce ATP to levels low enough to be insufficient to support cell viability. The insight provided by the findings noted above and in Fig. 3 suggests that biochemical modulation to further depress ATP to still lower levels than that induced by the anticancer agent alone would kill these sublethally injured cells, augment tumor regressions, and even yield some cures. The preclinical enhanced therapeutic results with MAP + anticancer agents support this thesis.

One new understanding of the paradox in obtaining improved therapeutic results by adding ATP-depleting modulatory treatment to the ATP-requiring apoptotic process is that necrosis and apoptosis are sometimes not completely separate entities in a cancer cell "hit" by an anticancer agent. Both modes of cell death are simultaneously induced by the DNA damage; more specifically, PARP activation as well as mitochondrial damage by a fall in the MPT (Fig. 3). If PARP cleavage occurs by activated caspase-3, necrosis is prevented and apoptosis prevails. If PARP cleavage is prevented by an IAP, necrosis prevails with an assist in ATP depletion from the apoptotically damaged mitochondria in the ongoing process of necrosis. It is understandable that ATP-depleting modulatory therapy would enhance necrosis and improve the therapeutic results. However, under conditions where PARP activity continues (*i.e.,* PAR synthesis and NAD^+ consumption continues), not only is there increased necrosis, but surprisingly, apoptosis also increases (58, 76). The latter situation (*i.e.,* uncleaved PARP leading to increased apoptosis) is not understood. Perhaps the continued activity of PARP induces changes in the pyridine nucleotide pool (NADH/NAD + NADPH/NADP) and nucleotide pool of ADP and ATP that regulate MPT (71, 79), leading to a fall in the MPT of additional mitochondria that affects rupture of these mitochondria-

releasing apoptogenic factors that result in increased caspase activity and increased apoptosis. Further research will hopefully explain the question mark in Fig. 3. If these conjectures apply, the MAP regimen (*i.e.,* its NAD^+ antagonist and ATP) could similarly influence the MPT and increase apoptosis.

ATP Depletion Is the Primary Mechanism of MAP.

Most pertinent to the question of whether the MMPR + 6-AN mechanism of enhancing ATP depletion has anything to do with enhancing tumor regressions is the demonstration that MMPR alone can reduce ATP levels to 34% in murine breast tumors, but in combination with 6-AN the ATP level is further reduced to 15% of normal (28). Importantly, this low level of ATP, 15%, cannot sustain cell viability (20, 21), and tumor regressions ensue. Also of relevance to ATP depletion and cell death, the combination of MMPR + 6-AN has been demonstrated to initiate a significant depletion of ATP prior to the onset of cell death (27).

There is published data (80) comparing both the therapeutic results and the ATP-depleting effect of MAP alone, MAP + FU, MAP + Adr, and MAP + FU + Adr. ATP depletion becomes more profound in conjunction with increasing levels of tumor-regressing therapeutic activity as treatment is increased from MAP, to MAP + FU or MAP + Adr, to MAP + FU + Adr; the latter levels of ATP depletion and tumor regression rates were significantly lower than that observed in tumors from mice treated with MAP + FU or MAP + Adr (80). Thus, a positive correlation was found between increasing levels of ATP depletion and increasing tumor regression. In other studies (81), both the depletion of ATP by MAP + Adr and tumor regressions were significantly greater than that of MAP alone. Thus, this correlative quantitative data supports ATP depletion as a significant factor in the production of tumor regressions.

The recent reports that blocking activated caspases by exogenous caspase inhibitors (Z-VAD-fmk; Refs. 22, 56, 64, 66–72, 77, 78) or endogenous inhibitors (IAPs; Ref. 52) prevents the apoptotic mode of cell death but causes the ATP-depleting form of cell death, necrosis, clearly demonstrate that ATP depletion can be made into a primary effector of cell death. Manipulation of cellular energy metabolism (*e.g.,* inhibition of the mitochondrial respiratory chain or provision or withdrawal of substrates for glycolysis) shifts the balance between apoptosis and necrosis (22). All of these shifts to death by necrosis are physiological effects attributable to severe ATP depletion; very low levels of ATP cannot sustain cell viability (20, 21).

Taken together, all of the above facts are compelling evidence that the enhanced antitumor effects observed in our studies are the result of ATP depletion. Similar therapeutic gains have been obtained by concomitantly administering MAP with nine different DNA-damaging agents that, although they damage DNA by different mechanisms, induce in common the same processes of apoptosis and necrosis that evoke ATP depletion. Hence, cancer cells sublethally injured because of the DNA-damaging agents will have various degrees of ATP depletion that can be further reduced by MAP to cell-killing levels. It seems clear that ATP depletion is the critical biochemical event common to the cell deaths induced by nine mechanistically different anticancer agents when given with MAP.

Mechanisms of action other than ATP depletion have been ascribed to MMPR and 6-AN. MMPR, as a single agent, is reported to act as an inhibitor of tumor vascularization but did not kill cancer cells or cause tumor regression (82). 6-AN, as a single agent, is reported to up-regulate the glucose-regulated stress protein, GRP 78, a finding associated with potentiation of cytotoxicity *in vitro* of certain anticancer agents; however, the effect of 6-AN on ATP depletion, which is the likely cause of the enhanced cytotoxicity, was not measured (83). Multiple mechanisms of action have been demonstrated for almost all anticancer agents. For example, doxorubicin has had at least nine mechanisms demonstrated, but the interaction with topoisomerase II is nevertheless considered the primary triggering event for cell killing through apoptosis (84). The primary mechanism of action for the enhanced antitumor effect obtained by MAP plus an anticancer agent is clearly severe ATP depletion.

Proposed Clinical Trial of MAP

A proposed clinical trial of MAP has potential for a treatment advance in cancer patient care. Single agent 6-AN has been administered in three Phase I clinical trials in patients with disseminated cancer (85–87), and these studies demonstrated that 6-AN toxicity takes two clinical forms, a low-dose, mixed B complex vitamin deficiency and a high-dose-dependent central nervous system toxicity. Of note in the early clinical studies, 6-AN was given daily, whereas the proposed clinical trial for MAP is an infrequent intermittent schedule every 2 weeks; this toxicity should be much less.

It is the preclinically proven, ATP-depleting modulatory concept that requires appropriate clinical exploration and not specific drugs. Thus, the clinical trial need not necessarily be done with the MAP regimen to prove the therapeutic value of the ATP depletion concept at the clinical level. However, the MAP regimen seems a reasonable first choice, not only for the basic scientific data and reasons already given, and the successful preclinical data with MAP, but because a MAP clinical trial could be completed in a relatively short time. All three of the MAP drugs have been independently evaluated clinically, and therefore, their toxicities and some schedules are known. Cancer patients have received MMPR + PALA combined in a single regimen with a concomitantly administered anticancer drug, FU (88). Thus, evaluating the MAP regimen in the clinic merely requires integration of 6-AN into the clinically established MMPR + PALA regimen. Clearly, less time would be required for evaluating MAP in the clinic compared with new agents.

Conclusions

(*a*) Preclinical *in vivo* tumor studies have demonstrated that a combination of ATP-depleting agents (that reduce tumor cell ATP levels to $<$15% of normal) administered with anticancer agent therapy markedly enhanced tumor regressions and can even produce cures.

(*b*) Because of the knowledge of the basic mechanisms effecting necrosis and apoptosis and their interrelationships, the correlation of MAP-induced ATP depletion with MAP-induced tumor regressions, and the marked enhancement of preclinical anticancer activity by the concomitant administration of MAP + nine mechanistically different anticancer agents, the total data merit a MAP trial at the clinical level.

(*c*) At the preclinical level, the therapeutic opportunity opened by modulation of NAD^+ and ATP levels merits further research. Other pharmacological manipulations may further improve the MAP regimen.

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