Combination of Arginine Deprivation with TRAIL Treatment as a Targeted-Therapy for Mesothelioma

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Abstract. *In the present study we present data to show that certain tumor cells including malignant pleural mesothelioma (MPM) cells do not express argininosuccinate synthetase (ASS), and thus are unable to synthesize arginine from citrulline. Exposure of these ASS-negative cells to the arginine degrading enzyme, arginine deiminase (ADI-PEG20), for 72 h results in significant increases in cleaved caspase-3. Importantly, this apoptotic signal is further strengthened by the addition of TNF-related apoptosis-inducing ligand (TRAIL). Using flow cytometry, we showed that the combination treatment (ADI-PEG20 at 50 ng/ml and TRAIL at 10 ng/ml) for 24 h resulted in profound cell death with 67% of cells positive for caspase-3 activity, while ADI-PEG20 alone or TRAIL alone resulted in only 10-15% cell death. This positive amplification loop is mediated through the cleavage of proapototic protein "BID". Conclusion: Our work represents a new strategy for treating patients with malignant pleural mesothelioma using targeted molecular therapeutics based on selected tumor markers, thus avoiding the use of potentially cytotoxic chemotherapy.*

Malignant pleural mesothelioma (MPM) is an aggressive tumor that originates from the mesothelial cells lining the pleura (1) and is characterized by unrelenting locoregional invasion and encasement of contiguous intrathoracic organs leading to death from cardiopulmonary failure (2). MPM is

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etiologically linked to occupational asbestos exposure (3, 4) and previous findings pose several genetic/cellular mechanisms involved in asbestos fiber carcinogenesis (5-7), although a direct underlying cause remains unclear. Regrettably, the majority of MPM diagnoses occur in advanced stages, as it is often asymptomatic (2) and radiological detection tools are ineffective (7) at early stages of the disease. As such, there is a significant associated mortality after MPM diagnoses, with median survival thereafter of approximately 8-12 months (7, 8). Moreover, even when detected in early stages, MPM is resistant to current multimodal therapies (2, 8-10), including radical surgery in combination with systemic chemotherapy and thoracic radiation. Such radical therapies only yield median survivals ranging from 11 months (stage III) to 30 months (stage I) (11), while advanced or surgically-unresectable MPM is treated with either supportive palliative care or systemic chemotherapy (cisplatin and pemetrexate) with a median survival of about 9 months (12). From a therapeutic perspective, considerable effort has been placed on testing novel anticancer drugs, such as histone deacetylase inhibitors, new combinations of commonly utilized chemotherapy agents or targeting receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR). Unfortunately, no significant improvement in survival has been reported (13), resulting in an urgent need for new treatment paradigms.

Our groups, as well as others, have demonstrated that certain cancers, such as malignant melanoma, hepatocellular carcinoma, or MPM are auxotrophic for the non-essential amino acid arginine due to the fact that they do not express argininosuccinate synthetase (ASS) (14-19), a key enzyme in the urea cycle that catalyzes the synthesis of arginine from citrulline (Figure 1A). Arginine deprivation has been shown to be selectively cytotoxic to ASS-negative tumors, while sparing ASS-expressing normal cells (20-22). A clinically applicable strategy of arginine deprivation is the use of the
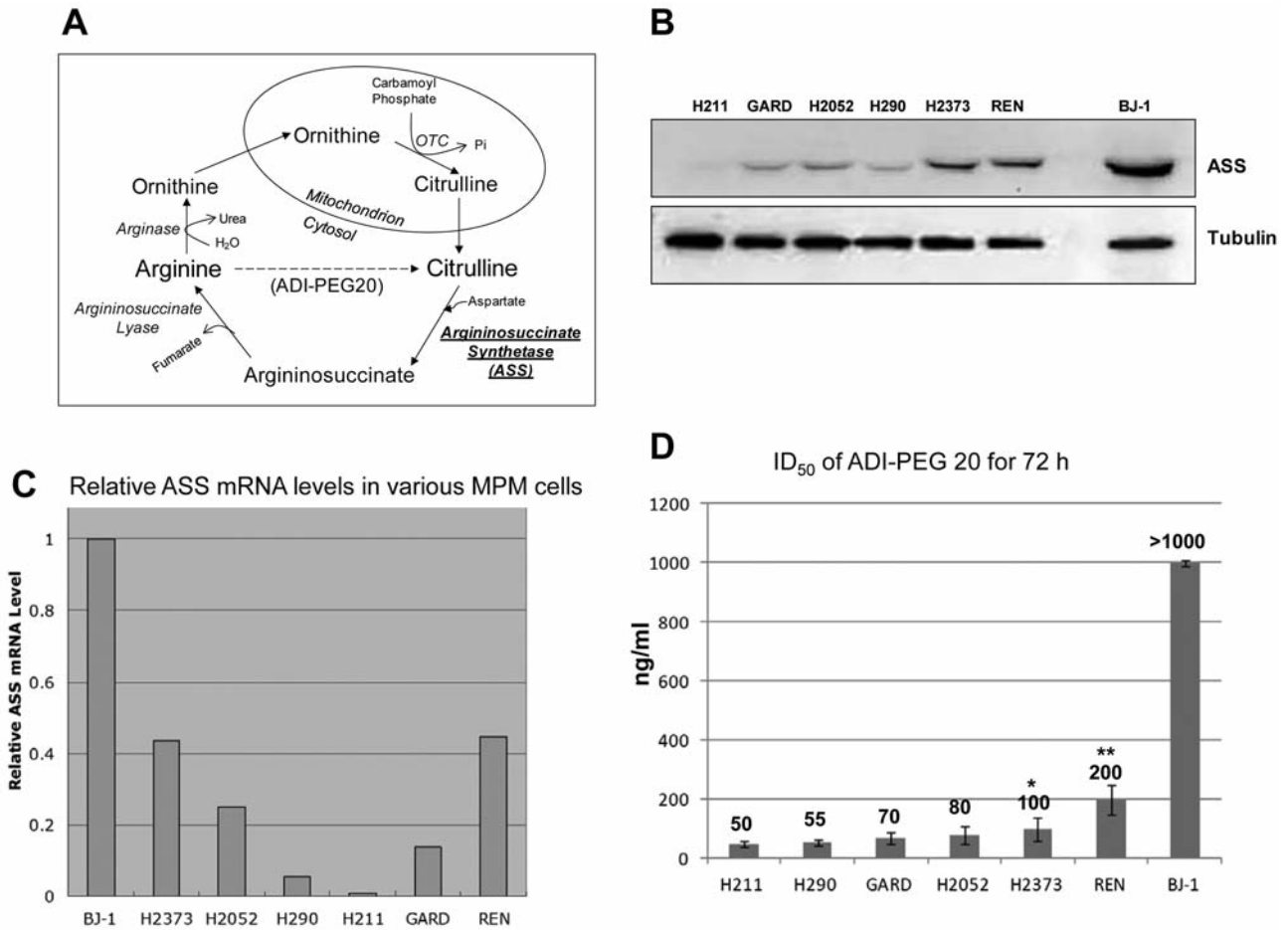
Figure 1. *ASS expression mediated the growth-inhibitory effect of ADI-PEG20. (A) Depiction of the arginine synthesis pathway (urea cycle). (B) ASS immunoblot of different MPM cell lines. BJ-1 was used as positive control for ASS protein expression. (C) Relative mRNA levels of ASS detected by quantitative real-time PCR of MPM cell lines. The critical threshold (Ct) of ASS mRNA was subtracted with Ct of GAPDH to obtain ΔCt. The ΔΔCt of MPM cell line was normalized from BJ1. (D) Growth inhibitory effect of ADI-PEG20 for 72 h in MPM cells, (*H211 vs. H2373: p-value<0.05, **H211 vs. REN: p-value<0.001), (Mean SD of 3 experiments).*

mycoplasma-derived enzyme arginine deiminase (ADI) that catalytically degrades arginine in the culture medium or in the blood plasma (23). The rapid depletion of arginine following ADI treatment *in vitro* results in selective cytotoxicity of ASS-negative cancer cells (14, 24). In fact, this concept has been translated into clinical application using a pegylated form of ADI (ADI-PEG20) to minimize antigenicity; therefore, increase its bioavailability and half-life (14). Partial response and stable disease have been reported by us and other investigators in melanoma and hepatocellular carcinoma patients treated with ADI-PEG20 in phase I/II clinical trials (12, 25-27).

A recent report indicated that up to 60% of cultured MPM cells and primary tumors have low or no ASS expression, thus making them susceptible to the growth-inhibitory effect of ADI-PEG20-mediated arginine deprivation (16). The

underlying mechanism of the ADI-mediated growth-inhibitory effect in susceptible cancer cells is complex and includes: cell-cycle arrest, activation of autophagy, and varying degrees of apoptosis (28). On this regard, it also has been reported that ADI-PEG20 can induce apoptosis in ASS-negative MPM cells *via* BAX activation that leads to mitochondrial inner membrane depolarization (16). Recently, we have demonstrated that ADI-PEG20 can induce the expression of extrinsic cell death receptors DR4/5, making cells susceptible to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Combining ADI-PEG20 with TRAIL resulted in significant cell death *via* an increase in pro-apoptotic protein (NOXA) expression (29).

In this communication, we report that ADI-PEG20 selectively inhibits cell growth in ASS-negative MPM cells and that the metabolic stress induced by arginine depletion hyper-sensitizes MPM cells to TRAIL-induced apoptosis.
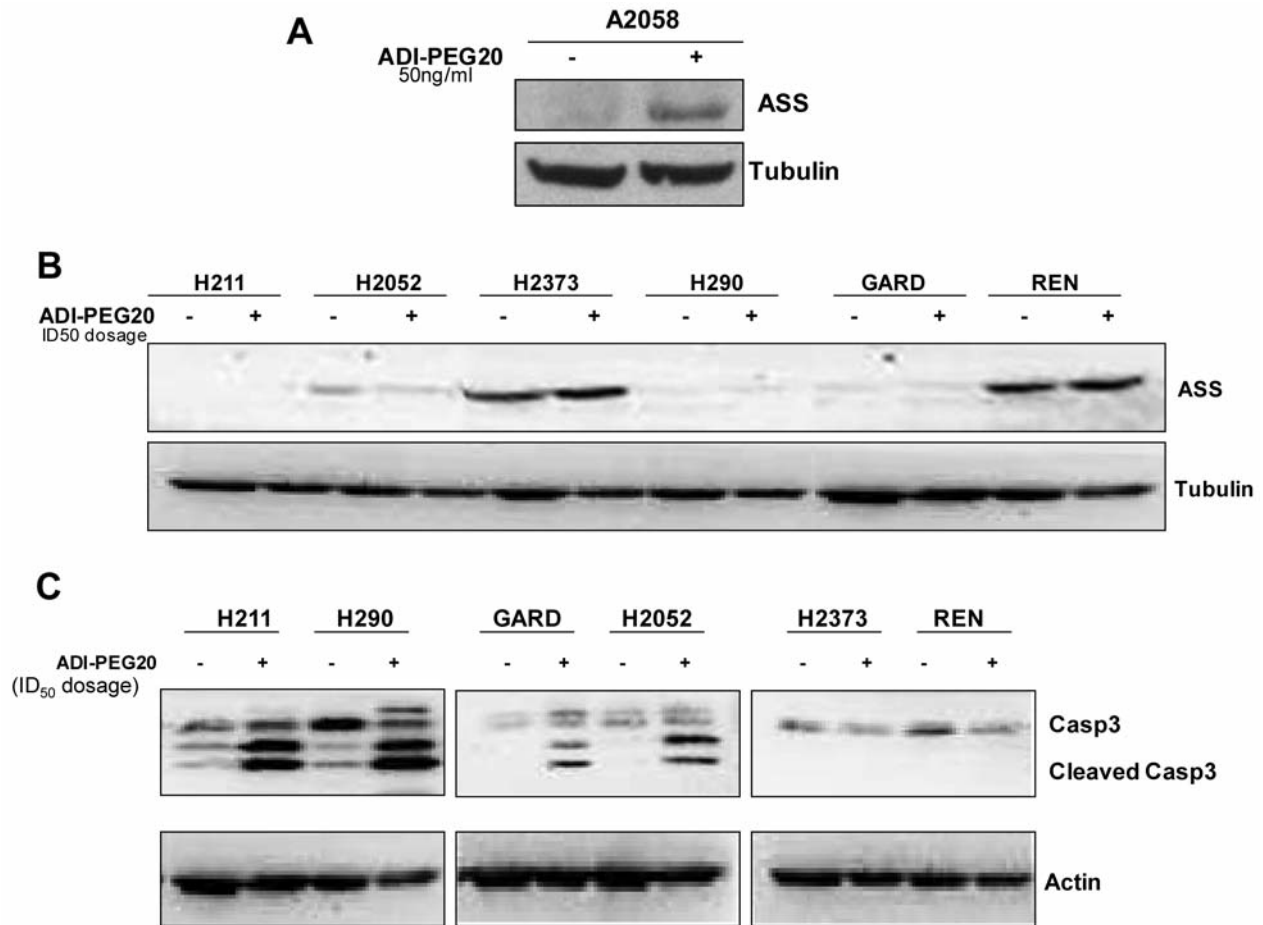
A

A2058

ADI-PEG20
50ng/ml
- +

ASS

Tubulin

B

H211   H2052   H2373   H290   GARD   REN

ADI-PEG20   - +   - +   - +   - +   - +   - +
ID50 dosage

ASS

Tubulin

C

H211   H290   GARD   H2052   H2373   REN

ADI-PEG20   - +   - +   - +   - +   - +   - +
(ID$_{50}$ dosage)

Casp3
Cleaved Casp3

Actin

Figure 2. *(A) ASS immunoblot in a melanoma cell line (A2058) showing that treatment of ADI-PEG20 for 72 h can up-regulate ASS protein expression. (B) ASS immunoblot of ASS in various MPM cell lines treated with ADI-PEG20 for 72 h. Treatment of ADI-PEG20 did not have an effect on ASS expression. (C) Immunoblot of cleaved caspase-3. Treatment of ADI-PEG20 for 72 h resulted in significant increase of cleaved caspase-3 in ASS^{Neg} and ASS^{+} MPM cells. ASS^{++} showed no change after exposure to ADI-PEG20.*

Materials and Methods

Cells and reagents. Cultured MPM cell lines: H211, H290, H2052, H2373, GARD and REN were generously provided by D.S. Schrump (NCI/NIH, Bethesda, MD, USA) and by H.I. Pass (New York University, New York, NY, USA). The immortalized fibroblast line BJ-1 was purchased from ATCC (Manassas, VA, USA). Clinic-grade ADI-PEG20 was kindly provided by Polaris Pharmaceutical (San Diego, CA, USA). The recombinant TRAIL was obtained from Genentech (Roche, San Francisco, CA, USA). ASS was purchased from BD Bioscience (San Jose, CA, USA). Cleaved caspase 3, caspase 9, tubulin, and BID antibodies were purchased from Cell Signaling Technology, (Danvers, MA, USA). Beta-actin antibody was purchased from Sigma (St. Louis, MO, USA).

Growth inhibitory assay. Cells were seeded in 24-well dishes and treated with various concentration of ADI-PEG20 for 72 h, as described previously (30). Briefly, at 72 h, the culture mediums as well as the trypsinized cells were collected and this mixture was centrifuged at $400 \times g$ for 5 min. The cell pellet was re-suspended in 1 ml of Hank's buffer and assayed for live and dead cells using the trypan blue exclusion method.

ASS expression by real time RT-PCR. qRTPCR was carried-out as previously described. One microgram of RNA was used for cDNA synthesis. Primers for ASS: 5'-CGCAATGACCTGATGGAG-3' (forward); 5'-TTCGTGTAGAGACCTGGAG-3' (reverse). *GAPDH* was used as an internal control. Primers for *GAPDH*: 5'-CTCTCTGCTCCTCCTGTTC-3' (forward); 5'-GGTGTCTGAG CGATGTGG-3'. All primers were custom-made from Sigma-Genosys. iQ SYBR Green Supermix (Bio-Rad; Hercules, CA, USA) was used for RT-PCR experiments. A 3-step (95˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec) and 40 cycle amplification was used. The reactions were carried out in a Bio-Rad iCycler PCR equipped with a MyiQ module. The data were analyzed with the iQ5 software from Bio-Rad. The level of ASS mRNA of each cell line was
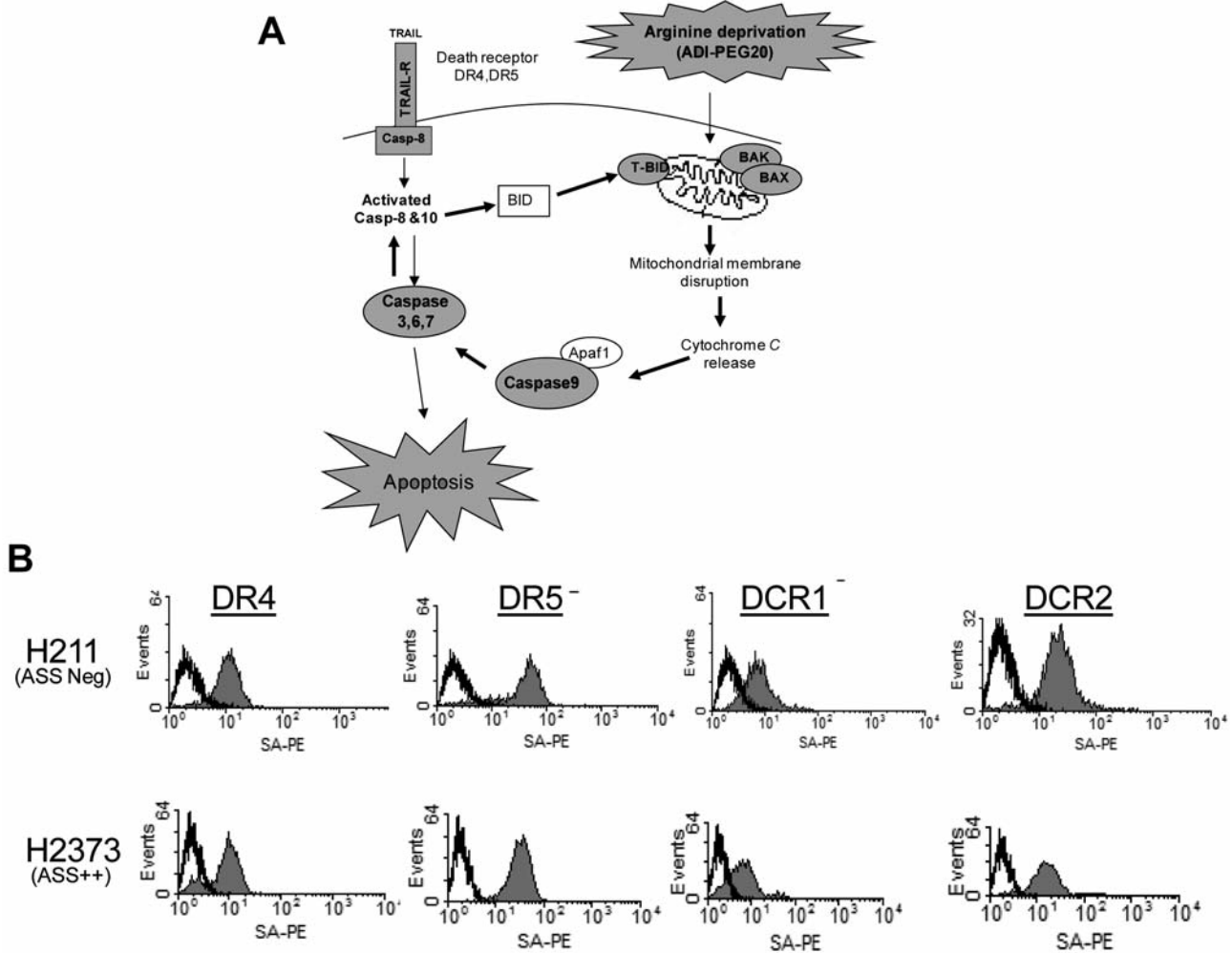
Figure 3. *(A) The extrinsic apoptotic pathway involves activation of death receptor by binding of trimeric TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) to its functional receptors DR4 and/or DR5 or to Fas receptor resulting in receptor aggregation. This in turn recruits FADD and pro-caspase-8 to the membrane to form the death-inducing signaling complex (DISC), leading to caspase-8 proteolytic cleavage and activation. The DISC-derived activated caspase-8 then cleaves and activates the executioner caspases-3 and -7, leading to apoptosis via a signaling cascade commonly referred to as the "extrinsic death pathway". It has also been shown that activated caspase-8 can cleave the BH3-only protein "BID" to yield truncated BID (tBID) that then translocates to the mitochondria to induce the oligomerization of Bax and Bak, resulting in the release of cytochrome c (Cyt c), thus linking the extrinsic to the "intrinsic death pathways". Activated caspase-9 cleaves caspase-3, which, can in turn, cleave and activate caspases-2, -8, -10 via activation of caspase-6 as an intermediary, thus forming a positive amplification apoptosis loop to further activate the initiator caspase-8. (B) Flow cytometry analysis of TRAIL receptor expression on the representative MPM cell line H211 and H2373. There are no differences in DR4, DR5 DCR1 and DCR2 expression in all the cell lines tested.*

adjusted with its respective *GAPDH* levels then normalized with BJ-1 cell line expression (the normalized value was set as 1). Briefly, the relative ASS mRNA level was calculated using the $\Delta\Delta Ct$ method. ΔCt=the difference in Ct values for the gene of interest and the endogenous control, *GADPH*. $\Delta\Delta Ct$=the subtraction of ΔCt control (BJ-1) from ΔCt of each MPM cell. The relative mRNA expression values then can be analyzed using $2^{\Delta\Delta CT}$.

Caspase activity. Apoptosis was analyzed with caspase-3 fluorescein-conjugated V-D-FMK (R&D systems; Minneapolis, MN, USA). Briefly, cells were processed in the same manner as in the growth inhibitory assays but, following centrifugation, the cell pellet

was re-suspended in 100 μl of staining solution (10 μl of casp-3 fluorescein + 20 μl of propidium iodide (PI) in 1 ml of PBS). The suspension was incubated at 37°C for 30 min. After the staining period, the suspension was washed once with 4 ml of PBS and centrifuged at 500 × *g* for 5 minutes to remove unbound reagent. The cells were re-suspended in 500μl of PBS and analyzed in a Coulter XL flow. A minimum of 10,000 cells were analyzed to generate caspase-3/ PI histograms.

siRNA. 8×10^5 cells were seeded in 60mm Petri dishes and incubated under normal culture conditions for 24 h. The dharmafect
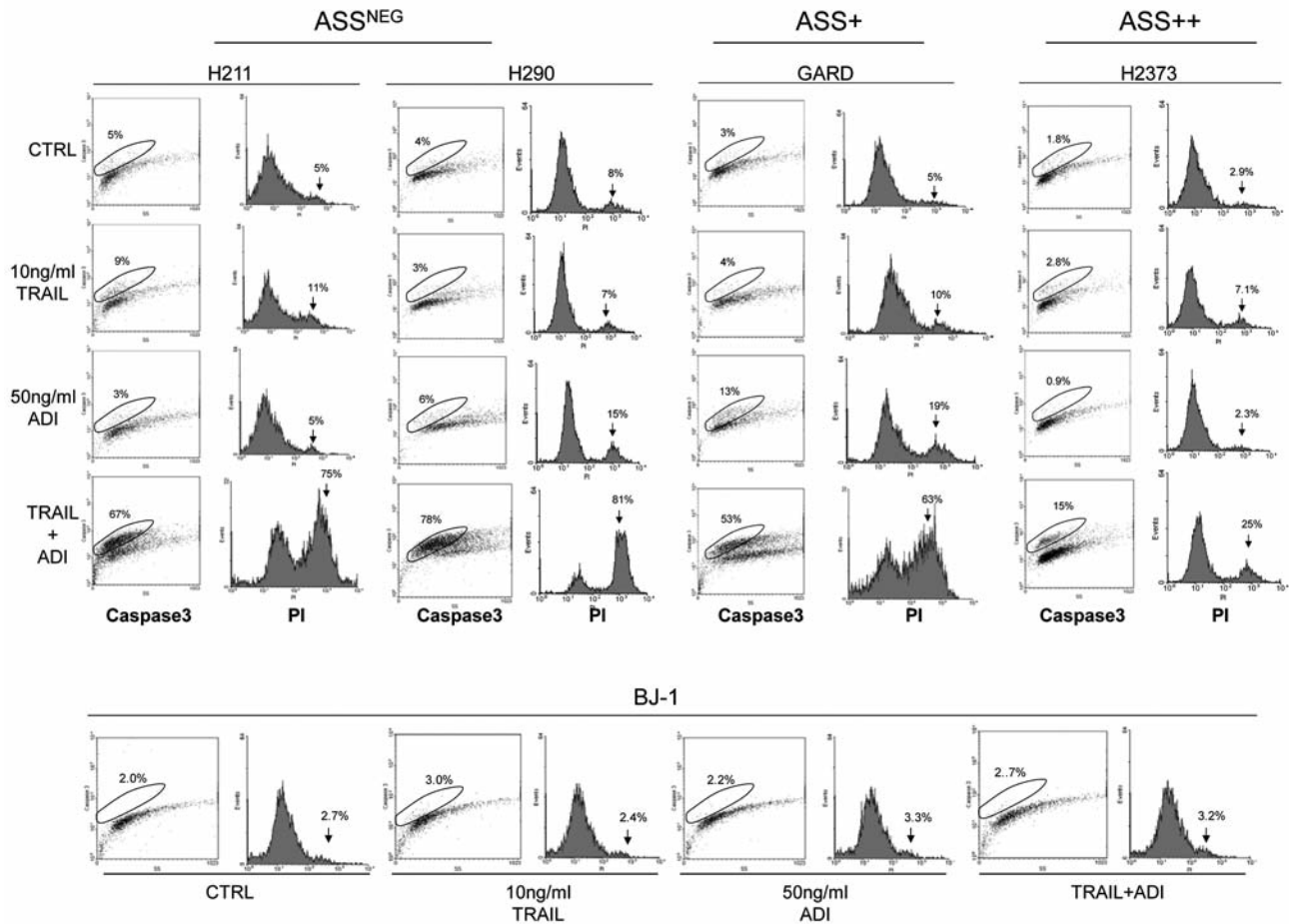
Figure 4. *ADI-PEG20 induces caspase activity in ASS^Neg and ASS^+ MPM cells. Cells were treated with TRAIL alone, ADI alone and in combination followed by caspase activity probe FMK-Fluoroscein and PI. The combination of ADI-PEG20 (50 ng/ml) and TRAIL (10 ng/ml) significantly increased caspase activity and PI when compared to either single treatment alone in ASS-negative and ASS^+ MPM cells. No significant increases in caspase activity were observed in ASS^++ (H2373) cells. Combination treatments also had no effect on normal cell fibroblasts, BJ-1. Each dot plot and histogram is representative of three experiments.*

1 transfection reagent was then used to transfect 100 nM of tBID-directed SMARTpool® siRNA or siCONTROL® (Dharmacon Co., Denver, CO, USA). The cells were incubated in the presence of transfection medium for 24 h under 5% CO_2 and 95% air at 37˚C. The transfection medium was then removed, cells were gently rinsed with PBS 3 times and fresh culture medium was replaced. Cells were then allowed to recover for 24 h under normal culture conditions and then trypsinized and reseeded either in 24 well plates or 100-mm Petri-dishes depending on the proceeding experimental protocols as described in the appropriate figure legends.

Western blots. As described previously (31, 32), cells were seeded at 1×10^5/ml onto 100-mm dishes, treated, collected and lysed. Total protein was separated on an SDS-PAGE, transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA) and immunoblotted with the indicated antibodies. The signal intensity was measured using a molecular imager Chemidoc system with Quality One software (Bio-Rad, Hercules, CA, USA).

Statistics and data analysis. All statistical analyses were performed from three separate measurements using the two-tailed *t*-test and the results were expressed as mean±standard deviation (SD). A *p*-value of less than 0.05 was considered statistically significant.

Results

ASS expression mediated the growth-inhibitory effect of ADI-PEG20 in a MPM cell line. The baseline expression of ASS in a panel of 6 cultured MPM cells lines was determined by immunoblots and qRTPCR assays using BJ-1 normal fibroblasts as a positive control (Figure 1B and C). Undetectable or very low levels of ASS expression were ascertained in H211 cells. We categorized these cells as ASS^Neg. Low to moderate expressions of ASS were found in H2052, GARD, H290, and hence categorized as ASS^+ cells. Conversely, highest levels of ASS expression were detected

in H2373 and REN and, therefore, categorized as ASS^{++} cells. *ASS* mRNA relative expression corresponded to ASS protein expression in the MPM cell lines, where ASS^{Neg} cells had *ASS* mRNA relative expression <0.05. ASS mRNA expression in ASS$^+$ cells ranged between 0.05-0.25 and ASS^{++} cells had ASS mRNA expression between 0.40-0.50. Three days of ADI-PEG20-mediated arginine depletion strongly inhibited the growth of ASSNeg cells. ADI- PEG-20 treatment was less effective in ASS$^+$ cells and had no effect on cell viability of ASS^{++} cells (Figure 1D). These findings were consistent with our previous reports, which showed that melanoma ASS^{Neg} cells are auxotrophic for arginine and undergo significant growth arrest after 3 days without arginine (33).

Arginine deprivation does not induce ASS expression in MPM cells. During our clinical trials, we came across certain ASS^{Neg} melanoma patients who developed resistance to ADI-PEG20 treatment. This resistance was due to the re-expression of *ASS* mRNA and ASS protein. In addition, we and others have shown that arginine depletion can induce ASS expression in ASS^{Neg} melanoma *in vitro* (Figure 2A) and other epithelial cancers. The re-expression of ASS was positively regulated by c-Myc/HIF1a (33). However, whether this phenomenon occurs in ASS^{Neg} or ASS$^+$ MPM cells is not known. The induction of ASS expression is an important factor preventing cellular apoptosis. Thus, in the absence of significant cell death, ADI-treated cells will recover and re-grow following withdrawal of arginine deprivation therapy. We exposed ASS^{Neg} and ASS$^+$ MPM cells to ADI-PEG20 for 3 days and assayed for ASS expression by Western blot. There were no detectable changes in ASS expression in these cell lines, indicating that arginine deprivation does not have an effect on ASS protein expression in these MPM cell types (Figure 2B).

Arginine deprivation induces apoptosis in ASS^{Neg} and ASS$^+$ MPM cells. To further elucidate the cellular mechanism of arginine deprivation-mediated cytotoxicity in susceptible MPM cells, both ASS^{Neg} and ASS$^+$ MPM cells were treated with ADI-PEG20 at the ID$_{50}$ dosage for 3 days and assayed for cleaved caspase-3. As shown in, Figure 2C, significant levels of cleaved caspase-3 were detected in ASS^{Neg} cells, as well as in ASS$^+$ cells after arginine deprivation. Caspase-3 cleavage was not observed in ASS^{++} cells, which further strengthen our hypothesis that ASS-positive MPM cells are not auxotrophic for arginine.

TRAIL enhances ADI-PEG20-mediated apoptosis in ASS^{Neg} MPM cells. We have reported that nutritional deprivation sensitizes melanoma cells to death *via* the intrinsic apoptotic pathway (34). Furthermore, we have shown that the apoptotic signal is further strengthened by the addition of death ligands, which induce cell death *via* the extrinsic apoptotic pathway, thus amplifying the apoptotic loop (Figure 3A). We then tested this concept in our MPM cells and found that MPM cells express abundant death-ligand receptors DR4/DR5 (Figure 3B). Herein we observed that combination of TRAIL and nutrient deprivation further increased ADI-PEG20 sensitivity in ASS^{Neg} and ASS$^+$ MPM cells (Figure 4). The combination treatment (ADI-PEG20 at 50 ng/ml and TRAIL at 10 ng/ml) for 24 h resulted in 75% (67% positive for caspase-3 activity) and 81% (78% positive for caspase-3) cell death in H211 and H290, respectively. ADI-PEG20 alone or TRAIL alone resulted in 10-15% cell death. Additionally, in ASS$^+$ (represented by GARD cells), there was 63% (57% positive for caspase activity) cell death in the combination treatment. TRAIL alone did not result in cell death, while 19% of cell death was observed in the ADI-PEG20 treatment alone. The enhancement effect did not occur in ASS^{++} (represented by H2373 cells) or in normal cells (BJ-1). These data clearly illustrated the degree of variability in efficacy of combination treatment in different ASS expressions MPM cell lines.

Combination of TRAIL and ADI-PEG20 treatment leads to further increased in tBID. Truncated BID (tBID) is an important pro-apoptotic marker that links the extrinsic and intrinsic apoptosis signaling pathways (Figure 3A). To confirm activation of the apoptotic loop, we treated MPM cells with ADI-PEG20 alone or in combination with TRAIL for 48 h and assayed for tBID (Figure 5A). We detected an increase in tBID protein in cells treated with ADI-PEG20 alone and further augmentation when combined with TRAIL in ASS^{Neg} and ASS$^+$ MPM. We were unable to detect tBID with TRAIL alone. Thus, our data suggested that the intrinsic death pathway is activated, to some extent, by ADI-PEG20 alone and addition of the extrinsic death signal ligand, TRAIL, can further increased the cleavage of BID. To determine whether cell death is enhanced through cleavage of BID, we transfected ASS^{Neg} cells with siRNA directed against BID. As shown in Figure 5B, siRNA against BID successfully reduced the protein levels of BID when compared with control transfectant. Following treatment with ADI-PEG20 alone or in combination with TRAIL, we found no evidence of truncated BID. Additionally, we also observed a decrease in the level of cleaved caspase 3 and 9, indicating attenuation of the apoptotic loop (Figure 5B). Furthermore, we performed a cell viability assay and found that siRNA against BID rescued ASS^{Neg} cells from the cytotoxic affect of ADI-PEG20+TRAIL combination, strongly suggesting that the enhanced apoptotic effect observed is mediated through tBID. The pan-caspase inhibitor, zVAD, also reversed the cell death seen in TRAIL+ADI treatment, which further indicated that these MPM cells underwent apoptotic cell death (Figure 5C).
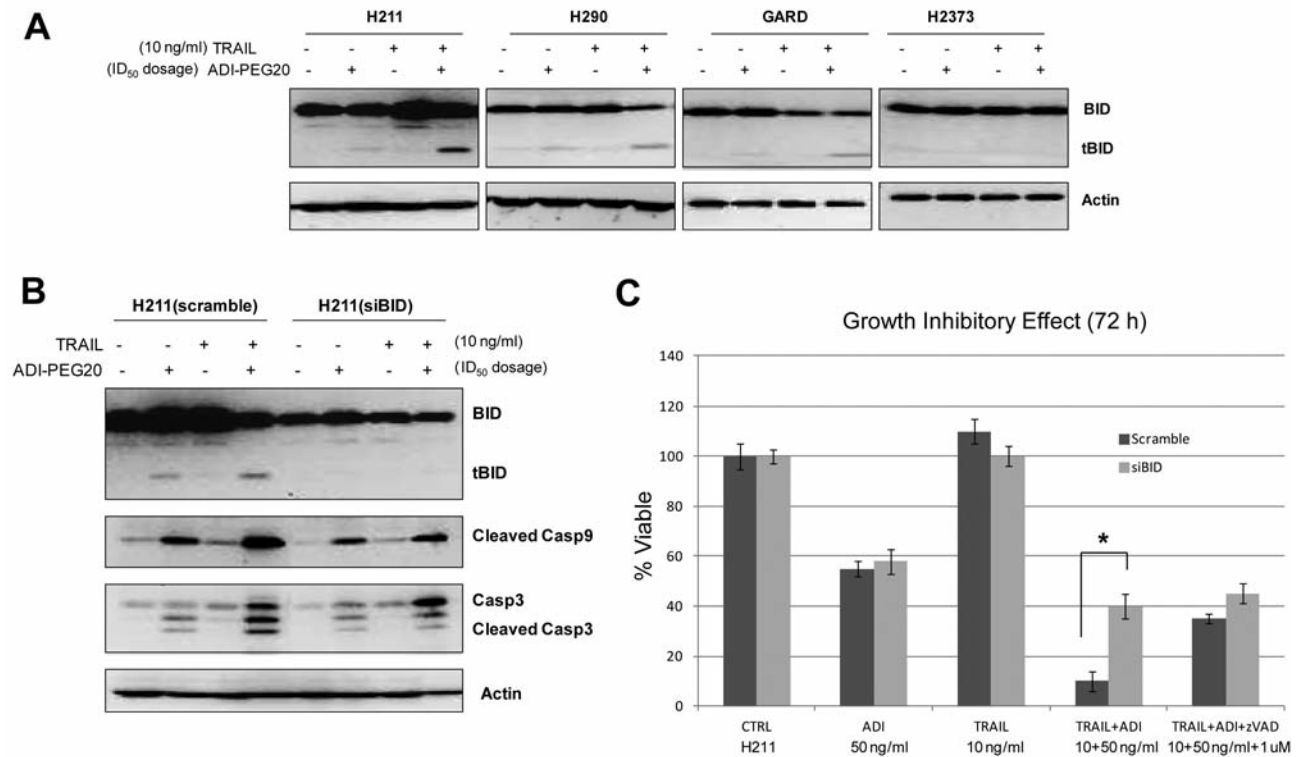
Figure 5. *Cleavage of BID in combination treatment was correlated with caspase activation. (A) Immunoblot of truncated BID (tBID). The combination of ADI-PEG20 and TRAIL increased tBID in ASS[Neg] and ASS^{+} MPM cells. Cleavage of tBID was not observed in ASS^{++} MPM cells. (B) Knocking-down BID attenuated caspase activity in treated ASS[Neg] MPM cells. H211 were transfected with scramble non-target siRNA (scramble) or siRNA targeting BID (siBID). (C) Growth inhibitory effect of treatment with ADI alone, TRAIL alone or in combination for 72 h. Down-regulation of BID resulted in decreased sensitivity of H211 to ADI+TRAIL. Treatment with 10 μM of zVAD also rescued TRAIL+ADI induced cell death, (*p-value <0.01, Mean SD of three experiments).*

Discussion

The majority of patients with MPM are diagnosed at locally-advanced stages. Patients with stage III/IV disease who are entered on therapeutic clinical trials have a median survival of less than 12 months and those with stage I/II, in whom aggressive multimodality therapy is instituted, have an overall median survival ranging from 12 to 30 months (35). Currently, the FDA-approved drug pemetrexed in combination with cisplatin offers a median survival of one year, with moderate side-effects (11). There is no approved second-line therapy for MPM patients who fail to respond to the cisplatin + pemetrexate combination. Clinical trials for MPM evolve around different combinations of cytototoxic chemotherapy, none of which includes novel molecularly-targeted agents. Thus, the effective treatment of MPM represents a serious unmet medical need that requires urgent attention with regard to the development of novel and effective therapy for this deadly disease.

It has been shown that 60% of MPM tumors do not express ASS, an essential enzyme to synthesize arginine from citrulline (16). Hence, cells lacking ASS (ASS[Neg]) require exogenous arginine to survive since arginine becomes the essential amino acid for their growth and survival. Following this finding, our group has shown that exposure of ASS[Neg] to arginine-free media or ADI-PEG20 results in growth inhibition and eventually leads to cell death both *in vitro* and *in vivo* (24, 36). However, ADI-PEG20 produces only partial responses and stable disease in ASS[Neg] patients, corresponding with our *in vitro* study, which demonstrated that certain ASS[Neg] melanoma cell lines readily undergo autophagy upon arginine deprivation (stable disease) and resume cell growth upon arginine repletion (28). Thus, the autophagic process appears to assist tumors in evading apoptosis upon arginine deprivation. Furthermore, in our clinical trial, we found that in ASS[Neg] patients, who had responded to ADI-PEG20 but later relapsed, re-expression of ASS was found in tumor samples taken at the time of relapse. In agreement with our clinical findings, we have also found that ASS can be induced in certain melanoma cell lines upon arginine deprivation. The re-expression of ASS is positively regulated by c-Myc, while HIF-1α negatively regulates ASS expression (33, 37). This re-

expression is due to the accessibility of c-Myc to interact with the ASS promoter without inhibition by HIF-1α. Moreover, a recent report by Szlosarek *et al*. showed that ASS regulation in MPM is taking place at the epigenetic levels, demonstrating that the molecule is actively regulated in these tumors (38). Our work adds an additional level of detail to ASS regulation in MPM and outcomes of regulation or dysregulation in the process of resistance to treatment and the apoptotic process. We report that ADI-PEG20-mediated arginine depletion inhibits proliferation of ASS[Neg] and ASS[+] without inducing ASS expression in these MPM cell lines. Thus, it is possible that ASS regulation is different among tumor cells and is most likely tumor type-dependent. Our data do support a model in which possible transcriptional (Figure 1D) regulation or different levels of *ASS* mRNA are found among different tumors and tumor types. These differences may be due to varying sensitivity to metabolic conditions or metabolic triggers, such as the deprivation from arginine. Additional investigation is warranted into the regulation of ASS protein expression in MPM through possible metabolic triggers and the subsequent effect on apoptosis or other treatment resistance schemes.

Our data clearly illustrated that ADI-PEG20-alone is able to induce cleaved-caspase-3 in 72 h. We have previously shown that arginine deprivation primes cells to undergo apoptosis by increasing DR4/5 (39). Thus, adding TRAIL to ASS-negative cells exposed to ADI-PEG20 greatly enhanced its cytotoxic effect within 24 h. Without the nutritional stress of arginine depletion by ADI-PEG20, MPM cells are not very sensitive to the cytotoxic effect of TRAIL alone. We also showed that combination treatments work in concert to cleave and activate tBID, which in part enhances the positive amplification apoptotic loop, thus increasing the efficacy of potential treatments. Knocking-down *BID* using siRNA can rescue ASS[Neg] cells from the cytotoxic affect of ADI-PEG20+TRAIL combination further suggesting that the enhanced apoptotic effect observed here is mediated through tBID. These data suggest that addition of TRAIL activated the extrinsic apoptotic pathway to some extent, resulting in the cleavage of BID (tBID). Truncated BID, in turn, activated the intrinsic pathway, which was already sensitized by arginine deprivation. Once the mitochondria are fully activated, this positive amplification feedback loop will be propagated resulting in more BID cleavage. Our findings further characterize certain MPM tumor types and add new molecular targets for combination or sequential tumor therapies that may utilize these apoptotic pathways to eliminate tumor cells while sparing normal cells.

Taken together, our data provide promising evidence for a novel treatment paradigm for MPM. Given that MPM represents a serious disease and since the lack of effective treatment is an unmet medical need, directed efforts with regards to the development of a novel and effective therapy for this deadly disease are necessary.

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