Supplementary Material

Arginine-Depleting Enzymes – An Increasingly Recognized Treatment Strategy For Therapy-Refractory Malignancies

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Table S1: Preclinical approaches to target Arginine-auxotrophic cancer.

Study setting/ Reference	Tumor entity/entities	Additional intervention	Treatment schedule	Methods	Outcome
in vitro [81]	HCC (Sk-Hep1, Huh7, Tong, HCC36, Hep3B, Malhavu, PLC5, Huh6, HepG2, SNU398, SNU182 Ovarian cancer (A2780, A2780CR)	Cisplatin	ADI-PEG20 (IC_{50}) Cisplatin (IC_{50})	- cell viability assay (CellTiter-Glo) - protein abundance (WB: ASS1) - MSP	- $ASS1^-$: ADI-PEG20 sensitivity, cisplatin resistance - methylation status of ASS1 promoter correlates with ADI-PEG20 sensitivity and cisplatin resistance - Cisplatin: down-regulates ASS1 in $ASS1^+$ HCC - ASS1 expression level during combination treatment predominantly dictated by ADI-PEG 20
in vitro [78]	$ASS1^-$ HCC (SNU398, SNU38), $ASS1^+$ HCC (HepG2,Huh-1) BJ-1 fibroblast	5-FU	ADI-PEG20 (0.1 µg/mL) and/or 0.1/0.2 µg/mL 5-FU	- proliferation assay (MTT) - gene expression (RT-PCR: *ASS1, OTC*) - protein abundance (WB: ASS1, XIAP, caspase-3, Mcl-1, Bcl2, TS, DPYD, PH-CAD - apoptosis/necrosis assay (flow cytometry (FC): Annexin V/PI) - immunohistochemistry (IHC): ASS1 (40 HCC patients)	- irreversible inhibition of thymidylate synthase - lethal DNA damage - mechanism: decline of survivin, Mcl-1, XIAP, Bcl-2 - increased apoptosis (Annexin V^+/PI^+; cleaved caspase-3) - BUT: Re-expression of ASS1
in vitro, in vivo [92]	Melanoma (A2058, SK-MEL-2, A375), Breast cancer (MDA-MB-231)	PI3K inhibitors (Ly294002, PI103), AKT inhibitors (VIII, perifosine)	ADI-PEG20 (0.3 µg/mL); LY294002 (5 µM), PI103 (1 µM), AKT inhibitor VIII (5 µM), Perifosine (5 µM) Xenografting (2×10^6 A2058 cells s.c.): ADI-PEG20 (4 IU/mouse), Ly294002 (25 mg/kg bw), combination	- siRNA transfection: transfected GSK-3 (α and β) - immunoprecipitation, Protein abundance (WB), and gene expression: c-Myc, Actin, p-S62-Myc, ERK, p-ERK, pGSK-3α, GSK-3α, pG Ras, total Ras, pT308-AKT, AKT, ERK, p-ERK, ASSK-3β, GSK-3β, p-T58-Myc - enzymatic activity assays: PI3K, PTEN - DNA fragmentation assay	- ADI-PEG20 induces c-Myc protein stabilization: a.) inhibition of ubiquitin-mediated protein degradation b.) mediated by ERK and PI3K/AKT-GSK3β signaling pathways c.) involvement of Ras signaling in c-Myc stabilization - *in vivo*: PI3K/AKT inhibition enhances ADI-PEG20-mediated cell killing via apoptosis + antitumor activity
in vitro [68]	Melanoma (A2058, SK-Mel-2, A375)	LY294002, mesylate, $CoCl_2$, Perifosine	ADI-PEG20 (5–10 IU/mg), LY294002 (IC_{50}), mesylate (50 µM), $CoCl_2$ (100 µM), Perifosine (IC_{50})	- establishment of ADI resistant cells - glucose uptake (radioactivity) - establishment of stable ASS1-overexpressing cell lines - protein abundance (WB: ASS1, c-Myc, HIF1α) - chromatin immunoprecipitation - proliferation assay (MTT)	- elevated *ASS1* expression in ADI resistant cells: c-Myc and HIF-1α interaction and mTOR downregulation - ADI resistant cells: a.) sensitive to PI3K/AKT inhibitors b.) enhanced *ASL* expression and glycolytic activities (*Warburg effect*) c.) sensitive to glycolytic inhibitors d.) elevated glutaminolytic metabolism and increased sensitivity to glutaminase inhibitors e.) c-Myc, but not elevated ASS1, contributes to the enhanced glycolytic and glutaminolytic metabolisms f.) expression of enzymes involved in lipid biosynthesis

in vitro [69]	ASS1$^-$ Melanoma (A375, A2058)	TRAIL inhibitor, Z-VAD-FMK), calpain inhibitor (ALLN)	ADI-PEG20 (0.1 g/ml)	- proliferation assay (MTT) - apoptosis/autophagy assay (Annexin V/FITC; GFP-LC3) - protein abundance (WB: Atg5, Beclin-1, LC3B)	- combined TRAIL and arginine deprivation: accelerated cell death: a.) TRAIL attenuated autophagic process during arginine deprivation b.) Beclin-1 and Atg5 cleavage
in vitro [93]	Melanoma (A2058, SK-Mel-2), embryonic kidney (HEK 293)	SAHA, HAT inhibitors (PU139, I-CBP112) CoCl2, MG-132	ADI-PEG20 (5–10 IU/mg), CoCl2 (150 µM), MG-132 (10 µM)	- siRNA and transfection (Lipofection: PHD2-A/B, LIMD1, p300, HDAC1/2, Sin3A) - immunoprecipitation, Protein abundance (WB), chromatin immunoprecipitation: ASS1, HIF-1α, pVHL, HA, c-Myc, PHD2, HDAC2, PCBP1, LIMID1, Sin3A, p300, hydroxy-HIF-1α, eIF2α, phospho-eIF2α, mTOR, phosphor-mTOR, p70S6K, 4EBP1, phosphor-4EBP1 - GST pull-down assay (GST–HIF-1α ODDD fusion) - *in vitro* hydroxylation assay (GST-ODDD) - proliferation assay (MTT) - apoptosis (DNA fragmentation assay)	- ADI-PEG20 induces: a.) HIF-1α degradation at ASS1 promoter b.) interaction between PHD2 and HDAC2 at ASS1 promoter c.) H3K14ac and H3K27ac deacetylation at ASS1 promoter d.) ROS to promote PHD2-HDAC2 interactions - reduced H3K14ac and H3K27ac levels in the matched patient-derived melanoma failed ADI-PEG20 treatment - combination: enhanced cell killing capacity
in vitro [94]	Mesothelioma (H211, H290, H2052, H2373, GARD, REN); immortalized fibroblasts BJ-1	TRAIL	ADI-PEG20 (0-1200 ng/ml) TRAIL (10 ng/ml)	- growth inhibitory/Apoptosis (Caspase activity) - gene expression (RT-PCR: *ASS1*) - siRNA - protein abundance (WB: Cleaved caspase 3, caspase 9, BID)	- no ASS1 re-expression upon ADI-PEG20 - mechanism: a.) apoptosis in ASS1$^-$ and ASS1$^+$ cells b.) TRAIL enhanced ADI-PEG20-mediated apoptosis in ASS1$^-$ cells c.) combined TRAIL and ADI-PEG20 treatment: further increased apoptosis
in vitro [27]	Primary GBM cultures + cell lines (DBTRG, GAMG, SNB19, U87, U118, CCF, LN229, 8MG, T87G, MO59J, MO59K, 42MG	5'AZA Z-VAD-fmk Chloroquine	ADI-PEG20 (0–2 µg/ml) 5'AZA (5 µM) Z-VAD-fmk (50 µM) Chloroquine (10 µM)	- IHC (ASS1, ASL, Iba-1) - gene expression (qPCR: ASS1) - protein abundance (WB: ASS1, autophagy antibody sampler kit) - DNA Methylation analysis (MSP and quantitative pyrosequencing: 5'AZA; ASS1 + ASL CpG islands) - caspase/autophagy inhibition assay - *ASS1* knockdown - generation of stable GFP-LC3 cells - phenotyping (FC: CD133)	- ASS1 promoter methylation: a.) *ASS1/ASL* silencing (10/22 cell lines, each) b.) autophagy upon arginine deprivation c.) predictive biomarker in GBM - knock down confirms role of *ASS1* in ADI-PEG20 sensitivity - arginine auxotrophic GBM CSCs: ADI-PEG20 sensitive - chloroquine inhibits autophagy, accelerates effects - caspase inhibitors do not prevent ADI-PEG20-induced cell death - *ASS1* and *ASL*: frequent targets for epigenetic inactivation in clinical GBM cases
in vitro, in vivo [82]	Patient-derived GBM (HROG02, HROG04, HROG05, HROG06, HROG07, HROG10, HROG13, HROG15, HROG17, HROG24, HROG36, HROG38)	temozolomide (TMZ), chloroquine, suberoylanilide hydroxamic acid (SAHA), Palomid 529	Recombinant *S. pyogenes* ADI (35 mU/ml) TMZ (0.125 mM, 0.375 mM) chloroquine (5 mM and 20 mM), SAHA (0.25 mM and 0.5 mM), MK0683), Palomid 529 (7.5 mM) Xenografting (HROG05 tumor pieces in NMRI Foxn1^{nu} mice): ADI (250 U/kg bw, twice a week), Palomid 529 (1 mg/kg bw, twice a week), SAHA (25 mg/kg bw	- production of *S. pyogenes* ADI and activity determination - gene expression (qPCR: *ASS1, ASL*) - MSP (*ASS1, ASL*) - cell cycle analysis (Flow cytometric DNA analysis) - biomass quantification (crystal violet (CV)) - CMFDA proliferation assay (FC-CMFDA staining)	- cell line specific response to ADI - inhibited proliferation of residual GBM cells - *ASS1* and *ASL* expression and methylation profile of primary GBM cell lines - combination: boosted antitumoral effects - antitumoral effects: independent from apoptosis - *in vivo*: xenograft growth inhibition

			twice a week), combination 1 (ADI + Palomid 529), combination 2 (ADI + SAHA)		
in vitro [31]	Patient-derived GBM HROG02, HROG05, HROG63, HROG52	TMZ, sorafenib, Vincristine, Curcumin, Resveratrol	recombinant *S. pyogenes* ADI (35 mU/ml) TMZ (2.5 µM), sorafenib (2.5 µM), vincristine (1 nM), curcumin (1 µM), resveratrol (10 µM) simultaneous, sequential therapy irradiation (30 Gy)	- biomass quantification (CV) - gene expression (qPCR: ArgI, CPSI, OTC, Human Cellular Stress Responses PCR Array) - phenotyping (FC: CD29, Nestin, Foxp3) - senescence β-Galactosidase staining - autophagy assay (acridine-orange microscopy) - protein abundance (WB: LC3A/B, Atg3, Atg5, Atg12, Beclin-1) - radiotherapy (LD_{50} = 30 Gy)	- ADI treatment: a.) stress-induction, altered gene expression of genes involved in arginine biosynthesis b.) no significant changes in surface markers c.) autophagy and senescence induction d.) radiosensitizing effects e.) no resistance development after long-term treatment f.) simultaneous vs. sequential ADI-based therapy - Combination: Boosted cell stress and autophagy (ADI + Resveratrol)
in vitro [11]	normal lymphoblastoid B-cell (NcNc), diffuse large B-cell lymphoma (DLBCL, Karpas-422), cutaneous T cell lymphoma (MyLa, SeAx)	5-Aza-dC, Chloroquine, doxorubicin	ADI-PEG20 (6.7 IU/ml) 5-Aza-dC (0.5–5 µM), Chloroquine (25 µM), doxorubicin (1 μM)	- IHC (ASS) - MSP - vi-Cell cell viability analyser - cell cycle analysis (Flow cytometry) - gene expression (qPCR: ASS1) - protein abundance (WB: ASS, (un-)cleaved PARP, pro-caspase 3, cleaved caspase 3, LC3I/II)	- ASS1 protein absent in most normal and malignant lymphoid tissues (methylation in malignant but not normal cells and tissues) - ADI-PEG20: a.) Reduced viability of *ASS1*-methylated lymphoid cells b.) Sensitivity dependent on *ASS1* promoter methylation c.) Caspase-dependent apoptosis in *ASS1*-methylated lymphoid cell lines d.) Enhanced apoptosis by Chloroquine - Clinical response to ADI-PEG20 in refractory lymphoma
in vitro [95]	(non)-/small-cell lung cancer (SCLC vs SR2), NCI-H460 vs H465CR, ovarian cancer (A2780 vs. A2780CP70; A2008 vs. A2008CR), GBM (A172 vs A172CR), melanoma (A2058)	Cisplatin, 5-Aza-dC, CoCl2, SAHA, bortezomib carfilzomib	ADI-PEG20 (0-4 µg/ml), SAHA (2.5 – 10 µM)	- Gene expression (qRT-PCR: c-Myc, ASS1, HIF-1α, DEC1) - Chromatin immunoprecipitation assay (ASS1 promotor-specific primers) - MSP and pyrosequencing - protein abundance (WB: ASS1, c-Myc, HIF-1alpha) - apoptosis (DNA fragmentation assay) - cell growth (sulforhodamine B (SRB) assay)	- *ASS1* suppression due to upregulation of HIF-1α and downregulation of c-Myc - DEC1 transcriptional regulator = master regulator of HIF-1α and c-Myc - Histone deacetylases modulate DEC1 and HIF-1α/c-Myc/ASS1 - correlation of *DEC1, HIF-1α, c-Myc* expression in tumor - Aza-dC induced-*ASS1* expression regulated by DEC1/HIF-1α/c-Myc axis - transcriptional start site and E-box sequences of *ASS1* unmethylated - enhanced ADI-PEG20 cell-killing: proteasome inhibitors for HIF-1α
In vitro, in vivo [80]	$ASS1^-$ melanoma (A375, Sk-Mel2, A2058 and Mel1220)	Cisplatin	ADI-PEG20 (0.05 ug/ml), cisplatin (0.1 µg/ml), combination Xenografting ($5x10^6$ A375 cells s.c. in Balb/c nude mice): ADI-PEG20 (53 IU/kg i.m. 6 days/week; (4 weeks total); cisplatin (6 mg/kg i.p.) once every 6^{th} day (3 doses total), combination	- growth inhibitory/apoptosis assay (Annexin V) - protein abundance (WB: NOXA, MCL1, BCL2, XIAP, SURVIVIN, FANCD2, FANCL) - immunofluorescence (γH2AX) - survivin knock down - gene expression analyses (microarray) - autophagy (Cyto ID staining)	- boosted growth inhibitory effect of cisplatin with ADI-PEG20 - increased tumor cell apoptosis - xenograft: similar growth inhibitory effects - mechanism: a.) enhanced DNA damage + decreased DNA repair b.) interference with apoptotic and anti-apoptotic proteins enhances antitumor effect
in vitro, in vivo [66]	Prostate cancer (LNCaP, RWPE-1 cells, PC3)	Paclitaxel, z-VAD-fmk, Chloroquine,	Paclitaxel (100 nM), z-VAD-fmk (50 µM), Chloroquine (25 µM), 2 µM	- ^{18}F-FDG PET imaging: 4 or 24 hours after ADI-PEG20 - gene expression (RT-PCR and qRT-PCR: ASS1)	- ADI-PEG20 sensitivity correlates with ASS1 expression - ADI-PEG20: a.) caspase-independent apoptosis/autophagy *in vitro*

	transiently transfected, CWR22Rv1 cells stably transfected with eGFP-LC3 plasmid	Rapamycin	rapamycin Xenografting ($1x10^6$ CWR22Rv1 cells s.c. in Balb/$c^{nu/nu}$ mice): ADI-PEG20 (225 µg/mL), docetaxel (10 mg/kg bw), combination	- protein abundance (WB: ASS1, caspase-3, Beclin1, pospho-AMP kinase, phospho-mTor, phospho-S6 kinase, phospho-S6, LC3, ERK1/2, phospho-ERK1/2, ELISA (Active Caspase-3) - proliferation assay (MTT) - apoptosis/necrosis assay (sub-G1 DNA fragmentation) - fluorescence Microscopy (LC3) - IHC on primaries (ASS1: 88 tumors and 59 normal samples)	b.) decreased tumor metabolic activity c.) ADI-PEG20 synergizes with taxane d.) autophagy delays and protects against ADI-PEG20-induced cell death - *in vivo:* a) ^{18}F-FDG uptake decreased after 24 hours b) tumor SUV decreased by 30 % after treatment: metabolic activity c) reduced tumor growth d) combination: synergistic effect
in vitro, in vivo [77]	Sarcoma (SK-LMS-1, MNNG/HOS, MG-63, SK-UT-1, SK-UT-1B, Fuji, RD-ES, LUPI, NOS-1, HuO 9N2, HCH-1, SK-ES cells) U-2 OS cells, ASPS-1 cells, 293 T, SYO-1 cells	Chloroquine 5-aza-dC Necrostatin ZVAD-FMK Pepstatin A E64D	Chloroquine (10, 20 µM), 5-aza-dC (5 µM), necrostatin (10 µM), ZVAD-FMK (100 µM), Pepstatin A (50 µM), E64D (25 µM) ADI-PEG20 (1 µg/ml) Xenografting ($1x10^6$ $ASS1^{low}$ MNNG/HOS cell or SK-LMS-1 cells, s.c. in nu/nu mice): ADI-PEG20 (320 IU/m^2, biweekly, i.m.); Chloroquine (60 mg/kg daily, s.c.), combination	- apoptosis/necrosis assay (FC: Annexin V/PI) - protein abundance (WB), co-immunoprecipitation (ASS1, LC3, p62, RIP1, caspase 8, BCL-2, cIAP1, cleaved PARP, caspase 3, RIP3, Atg5, Atg7) - IHC of the primary (ASS1)	- prevalence and prognostic value of $ASS1^{low}$ in sarcoma tumors and cell lines - ADI-PEG20: growth arrest - combination: ADI-PEG20 and chloroquine cause synthetic lethality via necroptosis
in vitro, in vivo [65]	Bladder cancer (RT112, 5637, SCaBER, 253J, T24, UMUC3)	Pemetrexed	ADI-PEG20 (750 ng/ml) Xenografting ($1x10^7$ UMUC-3 cells s.c. in $CD1^{nu/nu}$ mice): ADI-PEG20 (5 IU on days 1 and 8); pemetrexed (100 mg/kg on days 2–4 and 9–11), or combination	- ^{18F} (FLT)–PET imaging - IHC (ASS1) - MSP (ASS1 promotor) - ASS1 overexpression and knockdown - proliferation/Invasion assays - protein abundance (WB: ASS1, TS, DHFR, TK1, p53R2, PARP) - liquid chromatography–mass spectrometry	- loss of *ASS1*: poor survival in bladder cancer - methylated *ASS1* linked to increased proliferation and invasion of bladder cancer cells: sensitivity to ADI-PEG20 - ADI-PEG20 inhibits pyrimidine metabolism in *ASS1*-negative tumor cells - combination: enhanced effect
In vitro, in vivo [79]	Pancreatic cancer cell lines (MIA-PaCa2, PANC-1 ($ASS1^-$), L3.3 ($ASS1^+$)	Gemcitabine (GEM)	ADI-PEG20 (1.0 µg/ml) Xenografting ($1×10^6$ cells s.c. in athymic mice): ADI (5 IU, weekly), GEM (125 mg/kg, two days per week), combination	- intracellular amino acid levels - proliferation assay (MTT) - protein abundance (WB: RRM2, hENT-1, dCK, Caspase3) - cell cycle analysis (Flow cytometry) - transfections, reporter assays - apoptosis/necrosis assay (FC: Annexin V/PI) - IHC (DNA fragmentation (TUNEL), cleaved Caspase-3)	- ADI-PEG20: depletes intracellular arginine stores - *In vitro*: combination a.) caspase-3 cleavage b.) loss of Annexin V positivity c.) abrogated GEM-induced *RRM2* up-regulation - synergistically enhanced tumor growth inhibition *in vivo*

In vitro, in vivo [71]	Pancreatic cancer (MIA-PaCa-2, PANC-1, BxPC-3, SW1990), breast cancer (MDA-MB-453, BT474, MDA-MB-231, MCF-7) HCC (HepG2, MHCC97-H)	GEM	Recombinant *M. arginini* ADI (46 kDa) ADI (0–10 mU/mL) GEM (0 or 100 nM) Xenografting (1×10^7 PANC-1 cells in BALB/$c^{nu/nu}$ mice, s.c.): ADI (2 U/mouse), GEM (100 mg/kg), combination	- IHC/Immunofluorescence (ASS1) - gene expression (RT-PCR and qRT-PCR: ASS1, Caspase-3, Caspase-9, Survivin, Bax, Bcl-2) - protein abundance (WB: STAT3 [Tyr705], total-STAT3, p-ERK1/2 [Thr202/Tyr204], and ERK1/2, p-Akt [Thr308], total-Akt, total NF-κB p65, caspase-3, caspase-9, XIAP, c-Jun, p21, p53, survivin, p-c-Jun [S73], p-NF-κB p65 [S536], lamin B1, cyclin D1, ASS1) - proliferation assay (MTT) - colony formation assay (CV) - apoptosis/necrosis assay (FC: Annexin V/PI) - cell cycle assay (Coulter Epics XL flow cytometer) - NF-κB p65 nuclear translocation assay	- *ASS1*$^-$ associated with unfavorable outcome - ADI-PEG20: a.) cycle arrest (G1-phase), programmed cell death, attenuated colony formation ability b.) inactivates PI3K/Akt survival signal pathway c.) regulatory role on the expression of apoptosis-and cell cycle related genes, as well as phosphorylation of STAT3, AKT, and NF-κB d.) boosts GEM-induced cytotoxicity in pancreatic cancer cells/xenografts - combination: enhanced inhibition of survivin expression
in vitro, in vivo [96]	Embryonic kidney (HEK 293T/FT, breast cancer (MDA-MB-231, ATG5-, BECLIN 1-, SIRT3-knockdown MDA-MB-231, ASS1, SIRT3, GFP-LC3, MCF-7, ASS1-knockdown MCF-7, ZR-75-1, MCF-10A)	Bafilomycin A Mdivi1 Oligomycin FCCP	ADI-PEG20 (0-0.4 µg/ml) Xenografting (5×10^6 MDA-MB-231 and ATG5-knockdown MDA-MB-231 cells s.c. in NSG mice): ADI-PEG20 (4 IU/kg bw) weekly for 3 weeks	- IHC (ASS1) - gene expression (qRT-PCR, microarray) - cytotoxicity, apoptosis/necrosis assays (DIMSCAN assay, Annexin V/PI) - soft agar colony assays (MTT) - protein abundance (WB: MAP1LC3-I/II, SIRT3, Tom20, cyclophilin D, COX IV, ASS1, lenviral sh ASS1) - ATP assay (ENLITEN ATPAssay System) - oxygen consumption rate (Cellular mitochondrial function) - confocal microscopy/TEM/Oil Red staining	- ADI-PEG20: a.) selectively inhibits proliferation of *ASS1*$^-$ breast cancer cells b.) induces autophagy-dependent cell death c.) impairs mitochondrial bioenergetics d.) increases ROS and decreases mitochondrial membrane potential e.) induces mitochondrial fragmentation - autophagy required to cause shrinkage of ADI-PEG20 treated tumors *in vivo* - ASS1 abundance = prognostic factor for breast cancer survival
In vitro, in vivo	Myxofibrosarcoma (OH931, NMFH-1, NMFH-2) Dermal fibroblasts (CCD966SK) Human umbilical venous endothelial cells (HUVEC), Liposarcoma (LPS510: ASS1$^+$)	3-Dznep, UNC0638, Trichostatin A, 5-Aza-dC	Xenografting (OH931 myxofibrosarcoma cells transfected with plasmids expressing GFP-FFLuc in SCID mice): ADI-PEG20 (2.5 IU/kg bw),1 µmol/L 5-Aza	- stable transfection (lipofection: pASS1-DDK-Myc expression) - gene expression (RT-PCR: ASS1, EZH2, and angiogenesis-associated genes) - tumor characteristics (*ASS1* MSP and ASS1 immunostaining) - IHC (ASS1) - protein abundance (WB, ELISA) - functional assays (ASS1 re-expression, ASS1 knockdown, cell viability (BrdU assay), electric cell-substrate impedance sensing (ECIS), cell-cycle kinetic, soft-agar, HUVEC tube formation, wound healing and invasion assays) - *in vivo* imaging (IVIS camera system): monitoring of ADI-PEG20 treatment in xenografts	- *ASS1* differentially downregulated (methylation) - ASS1 IHC correlates with methylation status, clinicopathologic features, and patient survival (loss of ASS1 = worst outcome for disease-specific and metastasis-free survival) - ADI-PEG20 attenuates cell viability in *ASS1*-deficient cells - *In vivo*: a.) tumor shrinkage b.) fewer mitoses, increased collagenous matrix c.) less BrdU uptake in all *ASS1*-re-expressing cells - *ASS1* = tumor suppressor of myxofibrosarcomas - *ASS1* re-expression inhibits: a.) angiogenesis and tumor growth by suppressing cell proliferation (G1 arrest) b.) cell migration and invasion - Stable *ASS1* knockdown conferred proliferation and metastasis
in vitro, in vivo [7]	AML from patients	Cytarabine	Xenografting (in NSG mice): ADI-PEG20 (5 IU/mouse) once a week for 4 weeks,	- IHC (ASS1) - quantification of arginine and other amino acids (Ultra-high-performance liquid	- >90 % of cases: *ASS1*$^-$ - ADI-PEG20: a.) induces apoptosis in half of primary AML cells *in vitro*

			Cytarabine, 10 days at doses of 0.2 mg daily	chromatography mass spectrometry/mass spectrometry) - MSP (ASS1)	b.) depletes plasma arginine *in vivo* c.) reduces AML burden - resistant AML cells: increased *ASS1* expression - normal human bone marrow HSCs: enriched for ASS1 - ADI-PEG20–sensitive AML increased uptake of extracellular arginine via CAT-1 - AML-derived arginase depletes plasma arginine (not as effective as ADI-PEG 20)
in vitro, in vivo [97]	Different (RT4, SCaBER, UM-UC-3, T24, J82, Jurkat, RT112, 5637)	5-Aza-dC, chloroquine	ADI-PEG20 (1.2 µg/ml), 5-Aza-dC (0.3 µmol/L), chloroquine (25 µmol/L) Xenografting (1×10^6 cells in BALB/$c^{nu/nu}$ mice, s.c., right flank: RT112 cells ($ASS1^+$), left flank: UM-UC-3 cells ($ASS1^-$): ADI-PEG20 (5 IU/mouse)	- IHC on primary tumor tissue (ASS1) - protein abundance (WB: ASS1, p-eIF2α (Ser51;), eIF2α, GCN2, ATF4, (CHOP), LC3A/B, PARP, pro- and cleaved-caspases - transfection (Lipofection: ASS1 overexpression) - colony Formation Assay (CV) - proliferation assay (MTT) - apoptosis (Flow Cytometric Analysis Annexin V) - immunofluorescence (autophagy: LC3A/B) - IHC (DNA fragmentation (TUNEL))	- *ASS1* loss: evident in subtypes of bladder cancer, SCC, squamous cell carcinoma - ADI-PEG 20 a.) reduces colony formation and cell viability in ASS1-bladder cancer cells b.) induces Caspase-Independent apoptosis and autophagy c.) induces GCN2-dependent eIF2α phosphorylation (Ser51) and ATF4-CHOP induction d.) reduces *in vivo* tumor growth - modulation of *ASS1* expression: reverses response to ADI-PEG 20 in $ASS1^{-/+}$ cancer cells
in vitro, in vivo [91]	Different (A2780, AGS, A375, A549, HT-1080, MSTO-211H H1299, Mia Paca-2, SKOV-3, HT29, HCT116, SNU-398, SNU-16, Panc-1, K562, SNU-1 = ASS low), N87 = ASS1 high PBMCs from five different donors	IFN-γ anti-mPD-1	Xenografting (5×10^5 B16-F10 cells s.c. in C57BL/6 mice): ADI-PEG20 (12 mg/kg bw QWx2 i.m., anti-mPD-1 (10 mg/kg bw Q2D, i.p.) IFN-γ: 200 ng/ml	- cell viability and caspase 3/7 induction assay - phenotyping (FC: PD-L1 surface levels) - gene expression (RT-qPCR) - protein abundance - IHC (PD-1 and PD-L1)	- ADI-PEG20: cell line specific differences (concentration-dependent increase of PD-L1 in $ASS1^{low}$ cell lines) a.) affected by MEK/ERK and PI3K/AKT pathways b.) no effect on resting PBMCs but during initial stimulation: inhibited T cell activation c.) inhibit regulatory T cells d.) induces T cell infiltration in *in vivo* model e.) inhibits tumor growth in syngeneic models (combination with PD-1/PD-L1-neutralizing antibodies), combination boosted effects

CV – crystal violet; FC – Flow cytometry; GBM – Glioblastoma multiforme; GEM – Gemcitabine; IHC – immunohistochemistry; MSP – methylation-specific PCR; WB – Western Blot

Table S2: Overview on completed clinical studies using ADI-PEG20 alone or in combination with other substances.

Phase/ Study design/ Reference	Tumor entity/entities	Additional intervention	Treatment schedule	Treatment-related toxicities	Outcome
I [98]	9 patients with *ASS1*$^{-}$ cancers: 5 untreated patients with advanced malignant pleural mesothelioma (MPM) 4 untreated patients with stage IIIB or IV non-squamous non–small-cell lung cancer (NSCLC)	Cisplatin Pemetrexed	weekly: ADI-PEG20 i.m.: three patients each 18 mg/m^2, 27 mg/m^2, 36 g/m^{2*}, 48 hours before first drug dose every 3 weeks: cisplatin 75 mg/m^2 and pemetrexed 500 mg/m^2, both i.v.; daily folic acid 400 mg, i.m., hydroxycobalamin 1,000 mg, i.m., every 9 weeks, both started at least 7 days before the first dose. ADIPemCis therapy continued for maximum of six cycles (18 weeks)	no dose-limiting toxicities (DLT) adverse events (AE): grade 1 (83 % of total) or 2. most common toxicities: fatigue, nausea, vomiting, oropharyngeal toxicity (stomatitis, mucositis, and oral candidiasis), rash no DLTs or treatment-related deaths mostly attributable to pemetrexed and/or cisplatin therapy ADI-PEG 20: 9/38 patients: grade 1 or 2	rapid decline of circulating arginine levels, but rapid increase of citrulline levels; both changes persisted at 18 weeks ADIPemCis triplet prolonged overall survival vs. ADI-PEG 20 monotherapy in previous study (ADAM) Stable disease (SD) or better: all patients Partial response (PR): 7/9 patients (78 %) at all dose levels investigated, including three with either sarcomatoid or biphasic MPM
1/1B non-randomized, open-label [25]	9 patients without treated and 9 patients with untreated metastatic pancreatic cancer	Gemcitabine Nab-paclitaxel	weekly i.m. ADI-PEG20 injection of 18 (cohort 1: three patients) or 36 mg/m^2 (cohort 2: six patients) in combination with gemcitabine (1000 mg/m^2) and Nabpaclitaxel (125 mg/m^2) (both weekly for 3 of 4 weeks) Recommended phase 2 dose (RP2D): 11 patients (9 patients treated during the expansion phase and 2 patients treated in cohort 2 not receiving prior therapy)	Cohort 1: no DLT Cohort 2: 1 patient had a DLT (grade 3 elevation in bilirubin, aspartate aminotransferase, and alanine aminotransferase) All patients experienced grade 3/4 AEs (regardless of any drugs: 67 % neutropenia, 56 % leukopenia, 44 % anemia, and 33 % lymphopenia)	response rate in first-line setting: 45 % (5 months); median overall survival (OS): 11.3 months PR: 39 % (7 of 18); SD: 56 % (10 of 18), Disease Control Rate (DCR): 94 % (17 of 18) RP2D group: 45 % PR (5/11) SD: 45 % (5/11 for 8 or more weeks) DCR: 91 % (10/11) median duration of response: 3.7 months (1.9-11.0 months) progression-free survival (PFS): 6.1 months; OS: 11.3 months duration of arginine depletion to ≤10 µM in patients with PR and SD: 16 and 13 weeks median arginine levels suppressed and median citrulline levels elevated (4 week)
II Interventional [99]	21 patients with acute myeloid leukemia (AML), relapsed or refractory leukemia after receiving at least one prior therapy	None	weekly i.m. ADI-PEG20 36 mg/m^2 (4 weeks as one cycle) allopurinol to prevent hyperuricemia Treatment continued until progressive disease (PD), development of unacceptable toxicity, death, or withdrawal of consent for any reason	9 % grade 1/2 skin rash 7 % grade 1/2 hyperuricemia 7 % grade 3/4 leukopenia 5 % grade 3/4 neutropenic fever 5 % anemia One patient: grade 4 tumor lysis syndrome, infection, dead grade 4 anaphylactic shock in one patient	Complete remission (CR): 10 % (2/21), response durations of 7.5 and 8.8 months SD: 33 % (7/ 21) (3.5 months: median time to PD); PFS: 1.8 months; OS: 7.9 months DCR: 43 % correlated with median of 8 weeks (range 5–25) of arginine depletion to ≤10 µM or nearly undetectable in patients with CR or SD arginine level: markedly decreased, accompanied by increases in circulating citrulline levels after first dose some markers for AML subtypes and c-MYC

					regulated genes = ADI-PEG20 response predictors
I/II [100]	18 patients (advanced solid malignancies with a life expectancy greater than 3 months and with any number of prior therapies, but completed 4 weeks or more before study entry) nine: NSCLC three: castrate-resistant prostate cancer (CRPC), two or more forms of hormone therapy two: Head and neck squamous cell carcinoma four: small cell lung cancer, gastric cancer, colorectal cancer, thymic cancer	Docetaxel	<u>phase I</u> ADI-PEG 20: weekly i.m. injections; six patients at 4.5 mg/m^2; three at 9 mg/m^2; three at 18mg/m^2; six at 36 mg/m^2 dose level (dose escalated according to a standard 3×3 phase) Docetaxel: 75 mg/m^2, administrated every 3 weeks and 1 hr after ADI-PEG20 injection daily taken Prednisone (10 mg) by CRPC patients <u>phase II</u> study in HCC that demonstrated consistently higher citrulline levels (320 IU/m^2) ADI-PEG20: up to one year Docetaxel: up to 10 cycles	single DLT (grade 3 urticaria attributed to ADI-PEG 20), no additional DLT in remaining dose level hematologic toxicities: 14 patients (grade 3 to 4 leukopenia or neutropenia) non-hematologic toxicity fatigue (16 patients) Skin reactions (grade 3 or higher) including five rash, three palmar-plantar erythrodysaesthesia, and one urticarial rash Laboratory abnormalities: usually grades 1 to 2 (hypoalbuminemia), liver function test elevations and hyperuricemia, attributable to ADI-PEG 20	varying suppression of plasma arginine levels in 10 patients achieving at least a 50 % reduction of baseline values (longer suppression obtained at higher doses), reciprocal change in mean plasma citrulline levels return to baseline plasma arginine correlated with increased antibody titer CR: two patients (10 cycles of docetaxel, continued by ADI-PEG 20 alone) PR: four patients SD: 7 patients One patient with small cell lung cancer (low-dose ADI-PEG 20) treated for 67 weeks One patient with CRPC (high-dose ADI-PEG 20) still receiving treatment after completing docetaxel with 5 cycles of single-agent ADI-PEG 20
II randomized [101]	68 adults with advanced ASS1$^-$ MPM	None	Randomization 2:1 to arginine deprivation (ADI-PEG20, 36.8 mg/m^2, weekly i.m.) plus best supportive care (BSC) or BSC alone (median follow up: 38 (2.5-39) months)	AE: grade 3: 25 % (11/44) patients (ADI-PEG20) vs. 4/24 (17 %) patients (BSC) (P = 0.43) most commonly: immune related, fatigue, nonfebrile neutropenia, gastrointestinal events	*ASS1* gene-body methylation correlated with ASS1 IHC, longer Arg deprivation correlated with improved PFS. SD: 52 % (12/23) ADI-PEG20 vs. 22 % (2/9) BSC (P =0.23) at 4 months (modified RECIST) Median survival (MS): 15.7 ADI-PEG20 vs 12.1 months BSC (difference of 3.6 [95% CI, -1.0 to 8.1] months; P = 0.13)
I/II open-label [102]	31 patients with previously treated unresectable stage III or IV melanoma	None	phase I: weekly i.m. injections for 8 weeks in each of three dose levels including 40 (6 patients), 80 (6 patients) or 160 (19 patients) IU/m^2 ADI-PEG 20	Cohort 1 and 3: None Cohort 2: one case grade 3 arthralgia AE: grade 1 and 2: injection site pain, rash, and fatigue; treatment-related grade 3: 8 cases Maximum tolerated dose (MTD) reached eight patients (27.6 %, one in cohort 1 and 7 in cohort 3) had PMR after treatment initiation (not indicative of PR nor SD by RECIST criteria) ASS1 IHC: negative in 24 patients, 5 patients had <5 % positive cells	complete plasma arginine depletion: 97 % (30/31) by day 8, dose-independently Mean plasma ADI-PEG20 levels inversely correlated with ADI-PEG 20 antibody levels SD: 31 % (9/31): - 2 patients: durable SD for >6 months (in uveal melanoma) - 4 patients: uveal melanoma
			phase II (a Simon minimax two-stage design): 17 patients at MTD		→ SD rate: 67 % Improved median time progression for uveal melanoma patients (113 days *vs.* 57 days) PD: 71 % at first assessment phase II SD: 24 % (4/17), but: no objective response
II open-label, randomized	Asian patients with (pretreated) metastatic or locally advanced HCC; 88.7 % (63 patients) failed	None	ADI-PEG 20: weekly i.m. injections at doses of 160 (37 patients) or 320 (34 patients) IU/m^2 for 4 weeks (as one cycle) and maximum six cycles	AE: grades 1–2 local and/or allergic reactions and drug-related metabolic changes (26.8 % hypersensitivity/skin rash, 22.5 % local tissue reaction at injection site, 19.7 %	decreased circulating arginine level after 1st dose and gradual rise during treatment followed by reciprocal change in circulating citrulline levels, return to baseline plasma

[103]	previous therapies (40.8 % surgery, 64.8 % chemo-embolization, 43.6 % chemotherapy/ targeted therapy, 26.8 % radiation therapy)		3 and 5 patients in the 320 and 160 IU/m^2 arms: six cycles before ADI-PEG 20 administration: assessment of serum α-fetoprotein, uric acid levels, circulating arginine and citrulline levels; after every cycle (4 weeks): blood count, biochemistry and coagulation test, urinary analyses, anti-ADI-PEG 20 antibody titer	hyperuricemia, 15.5 % pruritus, 9.8 % fatigue, 4.2 % hyperammonemia, 7.0 % fever, 5.6 % diarrhea)	arginine correlated with increased antibody titer DCR and the OS: 31 % and 7 % - no statistical difference for both PFS and OS between two cohorts - patients with longer circulating arginine depletion (≥4 weeks) trend towards better OS
II randomized [75]	76 patients with metastatic or inoperable HCC with expected survival of ≥12 weeks 42.5 %: no prior treatment others: combination of HACE, systemic chemotherapy, radiofrequency ablation, i.t. ethanol, surgery	none	ADI-PEG20 weekly randomly administered i.m. 80 IU/m^2 (37 patients) or 160 IU/m^2 (39 patients) up to 6 months	AE: grade 1 or 2 (transient and reversible encephalopathy, skin irritation, or pain at the site of injection combined with low-grade fever) grade 3 or worse: 19.5 %	Mean survival: 15.8 months (474 days ± 39 days) from diagnosis, no significant differences between cohorts SD (according to the RECIST criteria) and DCR: 60 % and 63 % of patients CR/PR: 1 patient each Arg levels remained below baseline for 50 days while antibodies similarly reached a plateau Arg levels returned to baseline, the citrulline levels remained high patients with PD: higher increase in AFP levels after two treatment cycles
I and **II** [8]	Inoperable stage IV metastatic melanoma None of the patients had chemotherapy or surgery either 30 days before or during this study	none	15 patients in US phase I study: ADI-SS PEG: three i.m. injections at doses 20, 40, or 80 U/m^2 on days 1, 15, and 22 with three patients in each cohort; Six patients enrolled at 160 U/m^2 (OBD); 14 patients completed 24 patients in Italian phase I to II study: three i.m. injections of 40, 80, 160 or 320 U/m^2 on days 1, 15, and 22 (three patients in each cohort) Six patients: one cycle with 640 U/m^2 Six patients: three cycles (four once-weekly i.m. injections)	no grade 3 or 4 toxicities clinical laboratory abnormalities: hyperuricemia	- 160 U/m^2: decreased plasma Arg level (less than 2 µmol/L for at least 7 days) - US phase I study: no clinical response - Italian phase I to II study: - CR: one patient - PR: 21 % (5/24) with prolonged survival
I and **II** [104]	19 advanced or metastatic inoperable HCC patients None of these patients had any chemotherapy within 30 days before or during this study	none	three i.m. injections of ADI-SS PEG at dosages 20, 40, or 80 U/m^2 on days 1, 15, and 22 with three patients each Six patients: 160 U/m^2 Subsequent cycles: four treatments on days 1, 8, 15, and 22. Subsequent cycles initiated on day 36 of the preceding cycle 15 patients (79 %) completed all cycles	no evidence of systemic or local cutaneous allergic response, no SAE after treatment clinical laboratory abnormalities: hyperuricemia (adverse event) mean KPS: 66 % at start of therapy to 91 % at end the median Child-Pugh classification	160 U/m^2: decreased sufficient to lower plasma Arg from a resting level of approximately 130 µmol/L to below the level of detection (< 2 µmol/L) for more than 7 days Radiologic assessment indicated: CR: 11 % (2/19) confirmed by CT scans PR: 37 % (7/19) SD: 37 % (7/19) PD: 16 % (3/19) Mean durability of the response: >400 days (range, 37 to >680 days)

AE - adverse events; BSC - best supportive care; CR - complete remission; DCR - Disease Control Rate; DLT - dose-limiting toxicity; HACE - Hepatic arterial chemoembolization; HCC - hepatocellular carcinoma; IHC - immunohistochemistry; i.t. - intratumoral; MPM - malignant pleural

mesothelioma; MS - Median survival; MTD - maximum tolerated dose; NSCLC - non-squamous non–small-cell lung cancer OS - overall survival; PD - progressive disease; PFS - progression-free survival; PR - Partial response; RP2D - Recommended phase 2 dose; SD - Stable disease

Table S3: Overview on running or recently completed clinical studies using ADI-PEG20 alone or in combination with other substances.

Phase/Study design	Tumor entity/entities	Intervention	Treatment schedule	Status	Clinical trial.gov identifier
I Interventional	advanced gastrointestinal malignancies, HCC, gastric cancer, CRC	FOLFOX	not provided	recruiting participants	NCT02102022
I interventional	AML	Cytarabine	ADI-PEG 20 (18 and 36 mg/m^2) weekly in combination with low-dose Cytarabine (20 mg BID [twice daily] for 10 days, every 28 days)	recruiting participants	NCT02875093
I dose-escalation and interventional	NSCLC, advanced peritoneal mesothelioma, metastatic uveal melanoma, HCC, glioma, sarcomatoid cancers	Pemetrexed Cisplatin	Weekly ADI-PEG 20 at doses 18, 27 and 36 mg/m^2, pemetrexed 500 mg/m^2, Cisplatin 75 mg/m^2 both given every 3 weeks maximum of 6, 3-week cycles of ADIPemCis for a total of 18 weeks; NSCLC patients: 4 to 6, 3-week; patients completing ADIPemCis treatment may continue ADI-PEG 20 monotherapy if SD or better NSCLC patients may continue Pemetrexed + ADI-PEG20 and/or continue on ADI-PEG20 monotherapy after Pemetrexed	recruiting participants	NCT02029690
I	cutaneous melanoma, uveal melanoma, ovarian carcinoma	Cisplatin	not provided	completed	NCT01665183
I Interventional	HER2 negative metastatic breast cancer	Doxorubicin	single group assignment not provided	completed	NCT01948843
I interventional non-randomized	metastatic melanoma		ADI-SS PEG i.m. 3 times over 4-weeks 4 cohorts of patients each receiving a different dose level	completed	NCT00029900
I interventional	advanced HCC	Sorafenib	not provided	completed	NCT02101593
I/II, open-label, dose-escalation non-randomized (sequential assignment)	advanced melanoma		ADI-PEG 20, at escalating doses weekly i.m. for 9 weeks (cycle 1) or 8 weeks (subsequent cycles): - Cohort 1 (40 IU/m^2) - Cohort 2 (80 IU/m^2) - Cohort 3 (160 IU/m^2)	completed	NCT00520299
1B Dose escalation	advanced solid cancers	Pembrolizu-mab	ADI-PEG 20 (36 mg/m^2) weekly in combination with pembrolizumab (1 and 2 mg/kg or 200 mg) every three weeks	recruiting participants	NCT03254732
1B	advanced pancreatic carcinoma	Nab-paclitaxel Gemcitabine	not provided	ongoing	NCT02101580
II/III randomized parallel assignment	malignant pleural mesothelioma	Pemetrexed Cisplatin Placebo	ADIPemCis ADI-PEG20: 36 mg/m^2, i.m. weekly Pemetrexed: 500 mg/m^2 every 3 weeks Cisplatin: 75 mg/m^2, i.v. every 3 weeks	recruiting participants	NCT02709512
II interventional non-randomized	unresectable HCC		ADI-PEG20: 160 IU/m^2, weekly	completed	NCT00056992

II interventional randomized	unresectable HCC	Concurrent Transarterial Chemoembolization (TACE)	not provided ADI PEG 20 + TACE vs TACE alone	completed	NCT02006030
III interventional randomized double-blind	advanced HCC with failed prior systemic therapy	Placebo	ADI-PEG20: 18 mg/m^2, i.m., weekly until disease progression or toxicity Placebo: i.m., weekly until disease progression or toxicity	completed	NCT01287585

AML – acute myeloid leukemia; CRC – colorectal cancer; HCC – hepatocellular carcinoma; i.m. – intramuscularly; i.v. – intravenously; NSCLC – non-small cell lung cancer; TACE – Transarterial Chemoembolization