Appendix 1 Supplementary Methods

Immunoblotting analysis for protein expression

Cells were harvested at less than 70% confluence. The cells were then centrifuged at 800 × g for 5 min, washed with PBS, and lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA) containing 1% Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and 1% Phosphatase Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan). The lysate was centrifuged at 14,000 × g for 15 min. The supernatant was used as the protein lysate. The protein concentration in the lysate was measured using the Pierce Coomassie Plus Assay Kit (Thermo Fisher Scientific). Samples with an equal quantity of proteins were prepared by mixing the lysate with 25% Bolt LDS Sample Buffer (Thermo Fisher Scientific) and 10% Bolt Sample Reducing Agent (Thermo Fisher Scientific), and each sample was adjusted to an equal volume by adding distilled water and incubated at 95 °C for 5 min. The samples were electrophoresed on a Tris-glycine gel set in a Bolt Mini Gel Tank (Thermo Fisher Scientific). The proteins in the gel were transferred to a ClearTrans Nitrocellulose Membrane, 0.2 µm (Fujifilm Wako, Osaka, Japan), using a Mini Blot Module (Thermo Fisher Scientific). The membrane was rinsed with TBS and blocked with 5% non-fat skim milk or 2% ECL Prime Blocking Reagent (Cytiva, Marlborough, MA, USA) in 0.1% Tween 20 (Nacalai Tesque) in TBS (TBS-T) for 60 min, and incubated with a primary antibody diluted in 10% Blocker BSA in TBS (37520; Thermo Fisher Scientific) in TBS-T overnight at 4 °C. The membrane was washed thrice with TBS-T for 10 min and incubated with a secondary antibody diluted in 5% non-fat skim milk in TBS-T. The membrane was washed thrice with TBS-T for 10 min, and protein bands were visualized with ECL Select Western Blotting Detection Reagent (Cytiva) using Amersham ImageQuant 800 (Cytiva). The primary antibodies used for immunoblotting are listed in Table S3. ECL Anti-Rabbit IgG HRP-Linked Whole Ab Donkey (NA934; Cytiva, RRID: AB_772206) or ECL Anti-Mouse IgG HRP-Linked Whole Ab Sheep (NA931; Cytiva, RRID: AB_772210) were used as the secondary antibodies.

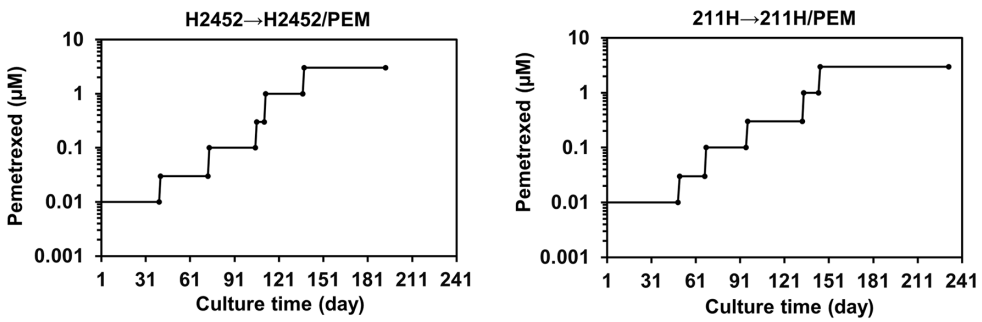

Figure S1 Establishment of two acquired pemetrexed-resistant MPM cell lines. H2452/PEM and 211H/PEM were established from human MPM cell lines H2452 and 211H by continuous exposure to pemetrexed. Dots indicate the pemetrexed concentration in the culture medium and the time point of pemetrexed concentration increase.

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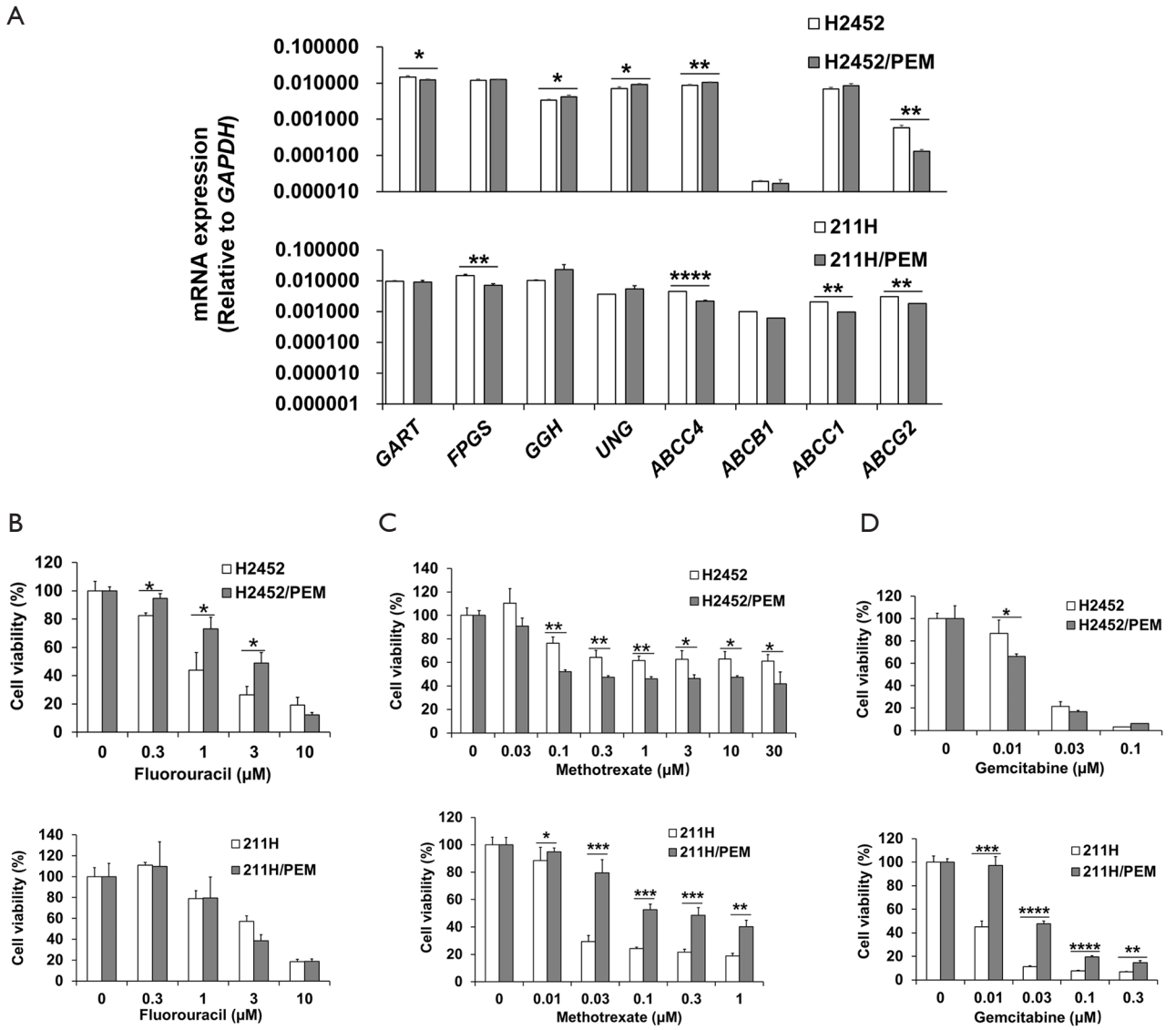

Figure S2 Evaluation of multidrug resistance to folate-related and anti-cytidine drugs of pemetrexed-resistant MPM cell lines. A, mRNA expression of genes related to folate and multidrug resistance proteins in MPM cells was detected by quantitative reverse-transcription PCR. Results represent the mean + SD. N = 3 (biological replicates). P values were calculated using the independent-sample *t*-test, *P<0.05, **P<0.01, ****P<0.0001. B, Viability of MPM cells treated with the indicated concentrations of fluorouracil for 120 h determined using the WST-8 assay. Results represent the mean + SD, N = 3 (biological replicates). P values were calculated using the independent-sample *t*-test, *P<0.05. C, Viability of MPM cells treated with the indicated concentrations of methotrexate for 96 (H2452) or 48 (211H) h determined using the WST-8 assay. Results represent the mean + SD. N = 3 (biological replicates). P values were calculated using the independent-sample *t*-test, *P<0.05, **P<0.01, ***P<0.001 D, Viability of MPM cells treated with the indicated concentrations of gemcitabine for 144 (H2452) or 48 (211H) h was determined using the WST-8 assay. Results represent the mean + SD, N = 3 (biological replicates). P values were calculated using the independent-sample t-test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

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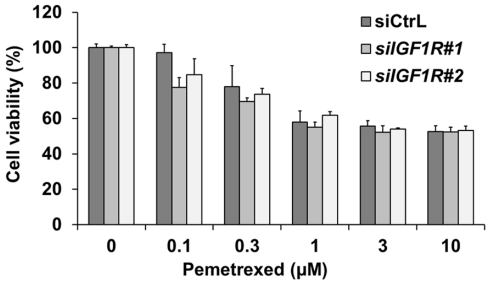

Figure S3 Viability of *IGF1R* knockdown pemetrexed-resistant MPM cells treated with the indicated concentration of pemetrexed for 96 (H2452/PEM) h. Negative control of siRNA transfection (siCtrl) was used. Results represent the mean + SD, N = 3 (biological replicates).

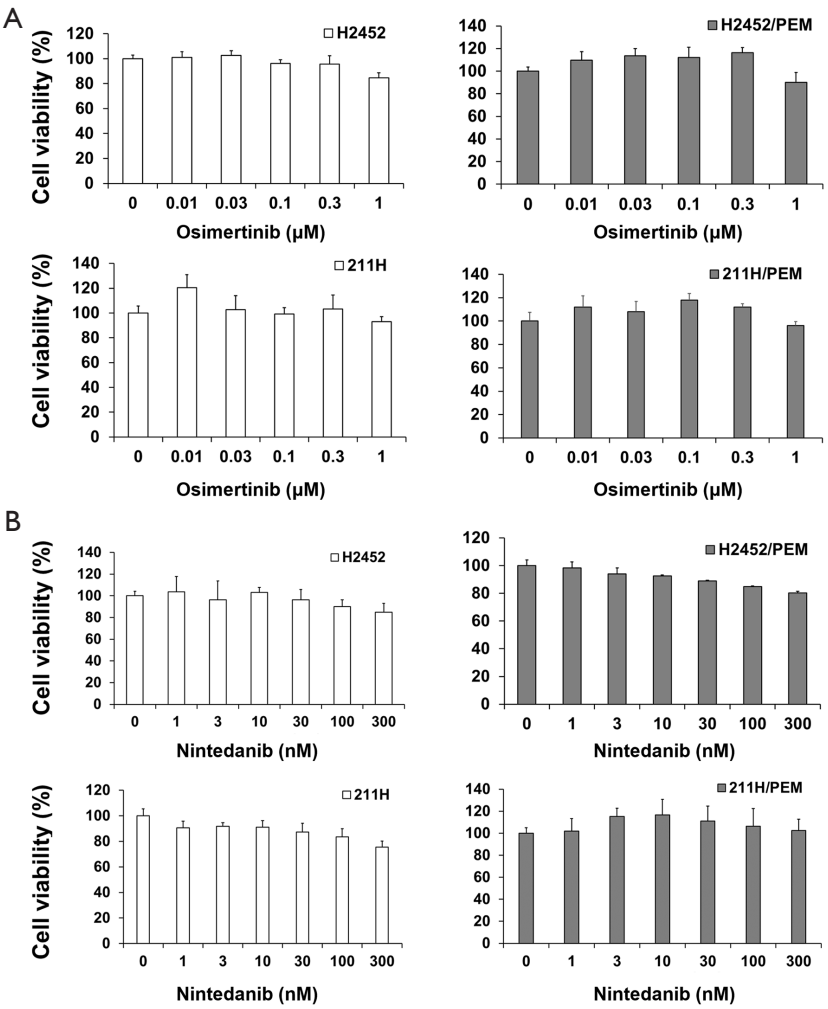

Figure S4 Acquired pemetrexed-resistant MPM cells were not sensitive to the other TKIs. (A) Viability of pemetrexed-resistant MPM cells treated with the indicated concentration of osimertinib for 120 (H2452) or 72 (211H) h determined using WST-8 assay. Results represent the mean + SD, N = 3 (biological replicates). (B) Viability of PEM-resistant MPM cells treated with the indicated concentrations of nintedanib for 144 (H2452) or 48 (211H) h determined using WST-8 assay. Results represent the mean + SD, N = 3 (biological replicates).

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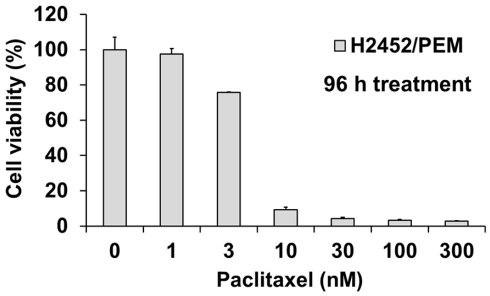

Figure S5 Viability of H2452/PEM treated with indicated concentrations of paclitaxel for 96 h determined using WST-8 assay. Results represent the mean + SD, N = 3 (biological replicates).

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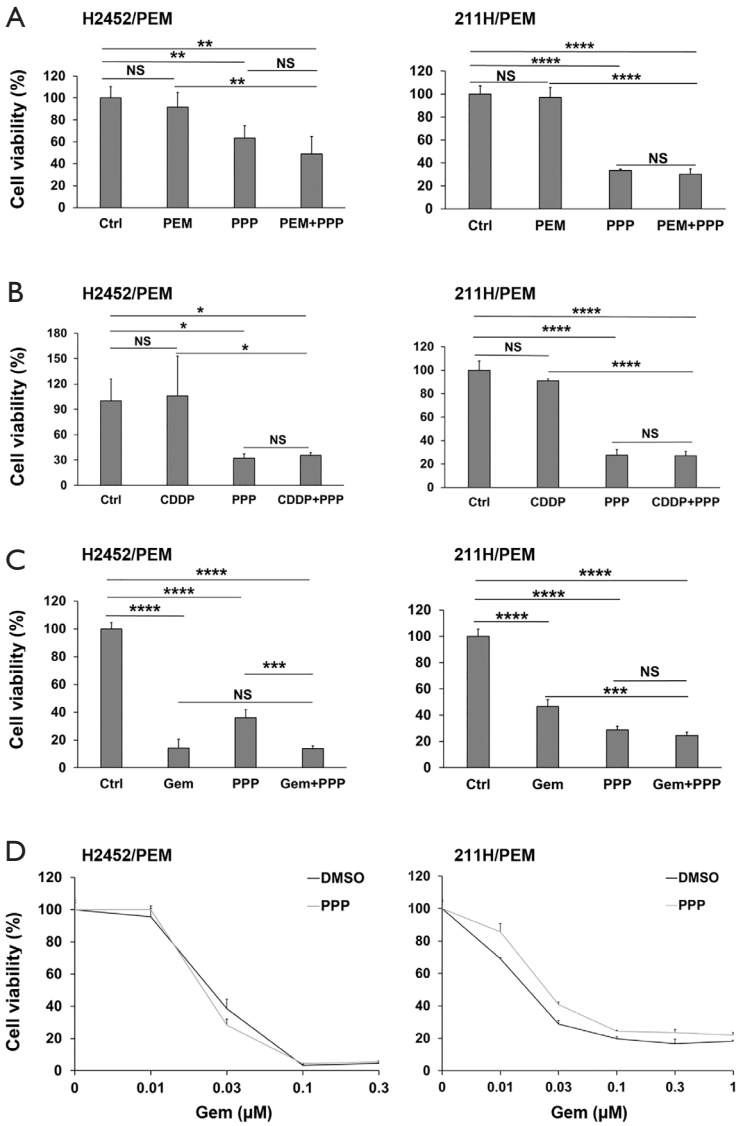

Figure S6 Assessment of picropodophyllin (PPP) plus other chemotherapeutic drugs on acquired pemetrexed (PEM) resistant MPM cells. (A) Viability of PEM-resistant MPM cells treated with 3 μM PEM without or with 0.5 μM PPP for H2452/PEM or 0.4 μM PPP for 211H/PEM and the same volume of DMSO as a control (Ctrl) for H2452/PEM (144 h) or 211H/PEM (96 h) determined using WST-8 assay. Results represent the mean + SD, N = 3 (biological replicates). P values were calculated using one-way ANOVA analysis and a post-hoc Dunnett's test, **P<0.01, ****P<0.0001, NS, not significant. (B) Viability of PEM-resistant MPM cells treated with 1 μM (H2452/PEM) or 0.7 μM (211H/PEM) cisplatin (CDDP) without or with 0.5 μM PPP for H2452/PEM or 0.4 μM PPP for 211H/PEM and the same volume of DMSO as a control for H2452/PEM (144 h) or 211H/PEM (96 h) determined using WST-8 assay. Results represent the mean + SD, N = 3 (biological replicates). P values were calculated using one-way ANOVA analysis and a post-hoc Dunnett's test, *P<0.05, ****P<0.0001, NS, not significant. (C) Viability of PEM-resistant MPM cells treated with 0.03 μM (H2452/PEM) or 0.01 μM (211H/PEM) gemcitabine (Gem) without or with 0.5 μM PPP for H2452/PEM or 0.4 μM PPP for 211H/PEM and the same volume of DMSO as a control for H2452/PEM (144 h) or 211H/PEM (96 h) determined using WST-8 assay. Results represent the mean + SD, N = 3 (biological replicates). P values were calculated using one-way ANOVA analysis and a post-hoc Dunnett's test, ***P<0.001, ****P<0.0001, NS, not significant. (D) Viability of PEM-resistant MPM cells treated with indicated concentrations of Gem without or with 0.5 μM PPP for H2452/PEM or 0.3 μM PPP for 211H/PEM and the same volume of DMSO as a control for H2452/PEM (144 h) or 211H/PEM (48 h) determined using WST-8 assay. Results represent the mean + SD, N = 3 (biological replicates).

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Table S1 Variants and copy number variations of tumor-associated genes

Gene	H2452	MSTO-211H
CDKN2A	loss	loss
CDKN2B	loss	loss
BAP1	A95D	wild type
LATS1	wild type	loss
LATS2	wild type	M785_L798del / M785I
MYC	wild type	Copy number amplification

Table S2 The primer sequences for quantitative PCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
DHFR	CCATACTGCTGAGATACAGGGAAAT	ACACAGGACAGGGAGCTGACA
GART	CAATGGCAGCCCGAGTACTTA	GACATGATGAGACTGTGCAAGTTTC
TYMS	CACACTTTGGGAGATGCACATATT	TTCGAAGAATCCTGAGCTTTGG
ABCB1	AGGCCAACATACATGCCTTC	CCACCAGAGAGCTGAGTTCC
ABCC1	CCTGTTCAACGTCATTGGTG	AGCCACGTAGAACCTCTGGA
ABCC4	TCTGGACCATCCGGGCATAC	TGGTGGTGGGCGTTTCTGAT
ABCC5	CCTGCAGTACAGCTTGTTGTTAGTG	GACACCGGTTCGGTAATTCAAT
FPGS	CTATGCCGTCTTCTGCCCTAAC	ACCTGGTCCAGTGTCACTGTGA
GGH	GCGAGCCTCGAGCTGTCTA	AATATTCCGATGATGGGCTTCTT
SLC19A1	CATCGCCACCTTTCAGATT	TGGCAAAGAACGTGTTGAC
IGF1R	TGGTGGAGAACGACCATATCC	CGATTAACTGAGAAGAGGAGTTCGA
PXN	ACGTCTACAGCTTCCCCAACAA	AGCAGGCGGTCGAGTTCA
ITGB1	CATCTGCGAGTGTGGTGTCT	AAGGCTCTGCACTGAACACA
GAB1	ATCAGAAACGCCAGCGAAGA	TCAGATACCACAAAGCACCA
GAB2	ACAGTACCTACGACCTCCCC	CTGGGCGTCTTGAAGGTGTA
JAK1	AGACTTGTGAATACGTTAAAAGAAGGA	AAAGCTTGTCCGATTGGATG
ERBB2	TGTGACTGCCTGTCCCTACAA	CCAGACCATAGCACACTCGG
PDGFRB	GCACCGAAACAAACACACCTT	ATGTAACCACCGTCGCTCTC
MET	CCATCCAGTGTCTCCAGAAGTG	TTCCCAGTGATAACCAGTGTGTAG
AXL	TACCGCCAGGGACGTATCGC	CCAGCACCGCGACATCAAGG
GAS6	TGGCGCGGAATCTGGTCATC	GAAGCACTGCATCCTCGTGTTC
EPHA2	CCGGCTACACTGCCATCGAG	GCCCAGCATCCCTGGTCATC
TYRO3	AACATCTTGGGCCAGCTGTCTG	GATTTGGTCAGTCCGGGCTTC
TWIST1	CATGTCCGCGTCCCACTAG	TGTCCATTTTCTCCTTCTCTGG
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

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Table S3 The information of primary antibodies for immunoblotting.

Target protein	Company	Catalog number	RRID
IGF1Rß	Cell Signaling	9750	AB_10950969
Phospho-IGF1R (Y1165+Y1166)	Bioss	bs-5449R	AB_11096251
Akt1/2/3	Santa Cruz	sc-8312	AB_671714
Phospho-Akt1/2/3 (S473)	Cell Signaling	4060	AB_2315049
Erk1/2	Cell Signaling	4695	AB_390779
Phospho-Erk1/2 (T202/Y204)	Cell Signaling	4370	AB_2315112
Thymidylate synthase	Agilent	M3614	AB_2210727
DHFR	Abnova	H00001719-M01	AB_565642
SLC19A1	GeneTex	GTX46753	AB_11174097
ABCC5	GeneTex	GTX81163	AB_11164308
Chk2	Cell Signaling	6334	AB_11178526
Phospho-Chk2 (T68)	Cell Signaling	2197	AB_2080501
PARP1	Cell Signaling	9532	AB_659884
alpha-Tubulin	Santa Cruz	sc-5286	AB_628411
GAPDH	Cell Signaling	8884	AB_11129865

IGF1R, insulin-like growth factor 1 receptor; DHFR, dihydrofolate reductase; SLC19A1, reduced folate transporter; ABCC5, multidrug resistance-associated protein 5; Chk2, serine/threonine-protein kinase Chk2; PARP1, poly(ADP-ribose) polymerase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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