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The solute carrier SLC35F2 enables YM155-mediated DNA damage toxicity

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Abstract

Genotoxic chemotherapy is the most common cancer treatment strategy. However, its untargeted generic DNA-damaging nature and associated systemic cytotoxicity greatly limit the therapeutic applications. Here, we employed a haploid genetic screen in human cells to discover an absolute dependency of the clinically evaluated anti-cancer compound YM155 on SLC35F2, an uncharacterized member of the solute carrier protein family that is highly expressed in a variety of human cancers. YM155 generated DNA damage through intercalation, which was contingent on the expression of SLC35F2 and its drug importing activity. SLC35F2 expression and YM155 sensitivity correlated across a panel of cancer cell lines and targeted genome editing verified SLC35F2 as the main determinant of YM155-mediated DNA damage toxicity *in vitro* and *in vivo*. These findings suggest a novel route to targeted DNA damage by exploiting tumor and patient-specific import of YM155.

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Author contributions

G.E.W. designed and performed experiments, analyzed and interpreted the data, made the figures and wrote the manuscript. B.R. designed and performed experiments, interpreted the data and prepared figures. C.M. performed mouse xenograft experiments, high-content microscopy and comet assay. V.B. helped performing the haploid genetic screen and conducted statistical analysis. C.T. performed RNA sequencing experiment and helped to perform MRM measurements. R.K.K. analyzed RNA sequencing data and performed statistical analysis. K.H. helped to set up MRM measurements and gave experimental advice. M.G. assisted with immunoblot analysis. D.C. created circos plot and graphical display of insertion sites. T.K. operated the next generation sequencer (Illumina HiSeq 2000) and helped with next-generation sequencing data handling. R.K. gave experimental advice and supervised next generation sequencing. S.K. gave experimental advice and designed experiments. O.F.C. designed mouse xenograft study, analyzed and interpreted data. T.R.B. codesigned study and gave experimental advice. G.S.F. codesigned and supervised the study and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Introduction

Cancer treatment strategies are facing a transition from the use of broadly cytotoxic substances to the employment of custom-designed agents capable of exploiting cancer specific molecular aberrations. Among others, tumor-selective targeting of otherwise generally toxic agents is a promising approach to reach the goal. While cell- and tissue-specificity can be achieved successfully by drug-antibody conjugations, comparable approaches focusing solely on small molecule compounds are rare.^{1–4} Systematic assessments of transport-cargo relationships in yeast have uncovered a pronounced dependency on active transport mechanisms of most tested small molecule agents.⁵ However, comparable relationships are less well understood in humans where the general involvement of transporter-mediated mechanisms in drug uptake as opposed to passive diffusion is a matter of active dispute.^{6–8}

YM155 (sepantronium bromide, Figure 1a) is a small molecule agent with *in vitro* and *in vivo* anti-tumor activity clinically evaluated for several malignancies including Non-Small Cell Lung Cancer (NSCLC), metastatic breast cancer and Non-Hodgkin's Lymphoma (www.clinicaltrials.gov).^{9,10} The precise mode of action is unknown. Among others, downregulation of the apoptosis inhibitor protein survivin, through binding to the interleukin enhancer-binding factor 3/NF110 transcription factor, has been suggested as mode of action of YM155.¹¹ Despite a remarkable potency in preclinical studies, the first results of clinical trials with YM155 as single agent have proven rather disappointing, possibly reflecting the uncertainty on the molecular nature of the target and the absence of a rationale for patient stratification.¹⁰

With the intention of elucidating the molecular basis of YM155-induced cytotoxicity as well as deciphering potential genetic roadblocks for its clinical efficacy, we devised a genomewide insertional mutagenesis approach in the near-haploid human cell line KBM7.¹² This screen allowed assessing gene-trap-mediated loss of function mutations that would rescue cells from YM155 induced cytotoxicity. We identified a single genomic locus, encoding an uncharacterized member of the 35F family of solute carrier proteins (SLC35F2), as capable of conferring drug resistance upon retroviral disruption. In the study that ensued we confirmed transport of YM155 by SLC35F2 and showed that its expression levels across a wide panel tumor-derived cells are the major determinant of sensitivity to the drug *in vitro* and *in vivo*. The complete dependence on intracellular availability found explanation in the elucidation of the primary mode of action of YM155 as a general DNA intercalating and damaging agent. The results generate the concept of a targeted induction of DNA damage for the treatment of cancer based on the expression levels of a single solute carrier gene.

Results

Haploid genetics reveals dependency of YM155 on SLC35F2

We mutagenized 1×10^8 near-haploid KBM7 cells with a retroviral gene-trap vector that inserts randomly into the genome. Insertions occur genome-wide, preferentially at actively transcribed genes, disrupt the genomic locus and can result in truncation of the expressed

protein product via a strong splice acceptor site.^{12,13} Subsequently, the complex mutagenized cell population was selected in the presence of 100 nM YM155 which resulted in the clonal outgrowth of resistant colonies. To identify mutations in genes causally linked to drug resistance, genomic DNA was isolated from the entire KBM7^{GT} (gene-trapped) pool and sequenced using an inverse-PCR based protocol that was described previously.¹³ We found enrichment for retroviral insertions in only one gene coding for the Solute Carrier Family Member 35 F2 (SLC35F2) with 122 independent insertion events mapping to that single locus (Figure 1b, 1c, Supplementary Table 1). In comparison to a large dataset of gene-trap insertions of a non selected KBM7 pool, this enrichment was highly significant (p -value = 7.58×10^{-299}).¹² Interestingly, there was no other locus in the genome that showed statistical significant enrichment for retroviral insertions, suggesting that SLC35F2 is, at the given drug concentrations, the dominant genetic determinant of drug sensitivity.

To validate the drug-gene interaction between YM155 and SLC35F2, two independent cell clones carrying gene-trap insertions in SLC35F2 were subcloned (further referred to as KBM7^{GT1} and KBM7^{GT2}). Successful disruption of the underlying genomic locus was shown via a nested PCR strategy (Figure 1d, Supplementary Results, Supplementary Figure 11). Transcript levels of SLC35F2 were below the limit of detection for both KBM7^{GT1} and KBM7^{GT2} compared to KBM7^{WT} cells (Figure 1e, Supplementary Figure 11). Thus, the retroviral genetrapp approach generated two isogenic loss-of-function alleles for SLC35F2. This allowed for testing the influence of SLC35F2 gene deletion on YM155 efficacy in a dose-dependent manner. After three days of drug incubation, we observed a more than 100-fold shift in the half-maximal effector concentration (EC_{50}) of YM155 in the clones deficient of SLC35F2 as compared to wild type cells. This shift was not observed when using nilotinib, a structurally unrelated control drug that exploits oncogene addiction by targeting the BCR-ABL oncogenic fusion kinase expressed in KBM7 cells (Figure 1f, Supplementary Figure 1a). To prove that the loss of SLC35F2 is causal for the observed resistance to YM155 treatment, we re-introduced C-terminal Flag-tagged SLC35F2 cDNA for stable expression in the KBM7^{GT1} clone (Supplementary Figures 1b, 1c, 11). The reconstituted clone (KBM7^{recon}) displayed a significant shift back towards higher sensitivity to YM155 whereas sensitivity to nilotinib remained unaltered (Figure 1f, Supplementary Figure 1a).

YM155 induces DNA damage in cells expressing SLC35F2

Next, we assayed dependence on SLC35F2 in the cellular response to YM155 treatment. We employed a RNA sequencing strategy to compare the transcriptional profile of KBM7^{WT} with that of KBM7^{GT1} cells upon six hour treatment with 1 μ M YM155 (Supplementary Figure 2a). KBM7^{WT} cells showed a clear transcriptional response (431 up and 404 down regulated transcripts, Supplementary Table 2) that was almost entirely absent in KBM7^{GT1} cells, the gene-trapped isogenic counterpart (Figure 2a, 2b). Monitoring the transcriptomic alterations for signatures of chemical or genetic perturbations as well as canonical pathways in a comprehensive geneset compendium (MSigDB)¹⁴, we identified DNA damage response as well as induction of apoptosis to be significantly enriched processes ($p < 0.001$) (Supplementary Figure 2b). Neither of these genesets was enriched in KBM7^{GT1} cells deficient of SLC35F2. To validate a putative DNA damage response caused by YM155

treatment, we treated KBM7^{WT}, KBM7^{GT1} and KBM7^{recon} with increasing concentrations of YM155 and assessed canonical DNA damage. We observed a dose-dependent increase in γ H2AX as well as phosphorylation of the ATM target KAP1 in KBM7^{WT} cells, indicative of the presence of DNA double strand breaks (DSB).¹⁵ Consistent with the toxicity data, this response was not observed in KBM7^{GT1} cells. As expected, reconstitution of KBM7^{GT1} with SLC35F2 cDNA fully re-established the dose-dependent induction of DNA damage by YM155 (Figure 2c, Supplementary Figure 11). To gain insight on the molecular mechanism of YM155 induced DNA damage, we assessed its properties to interfere with topological changes in plasmid DNA caused *in vitro* by topoisomerase treatment.¹⁶ In this assay, YM155 behaved similar to ethidium bromide, interfering with plasmid relaxation and thus locking the plasmid in a supercoiled state and at the same time, in contrast to the control compound camptothecin, not interfering with the action of topoisomerase. Thus, YM155 exhibits characteristics of a bona fide DNA intercalating chemical agent and not a topoisomerase I inhibitor (Figure 2d, Supplementary Figure 11).¹⁶ DNA intercalating agents such as chloroquine have been shown to interfere with DNA replication and to trigger H2AX phosphorylation.^{17,18} Interestingly, comet assays revealed that YM155 only generated DNA damage in a fraction of the treated cells (Supplementary Figure 3a, 3b), which prompted us to explore if this response was also linked to DNA replication. Accordingly, analysis of EdU incorporation rates revealed that YM155 inhibits DNA replication (Supplementary Figure 3c). Moreover, high throughput microscopy mediated analyses showed that the cell cycle distribution of γ H2AX is similar to what is observed with DNA replication inhibitors such as aphidicolin, the response being restricted to replicating cells (Supplementary Figure 3d). Altogether, these analyses provide mechanistic evidence for DNA being the primary target of YM155 action, consistent with the gene expression profile, the biochemical markers and the singular outcome of the primary genetic screen. Given that DNA breaks are highly cytotoxic we tested the ability of YM155 to induce apoptosis in the KBM7-based isogenic cell pair monitoring Annexin V and propidium iodide staining. The dose-dependent increase of apoptotic cells in KBM7^{WT} was largely absent in KBM7^{GT1} cells, whereas re-introduction of SLC35F2 reverted the phenotype. (Figure 2e, Supplementary Figure 4). In summary, these results indicate that YM155 is dependent on SLC35F2 for its capability to induce DNA damage and to cause apoptotic cell death.

SLC35F2 modulates the cellular uptake of YM155

SLC35F2 is a poorly characterized member of a family of solute carriers that facilitate the transport of nucleotide-sugars through biological membranes.^{19,20} As opposed to the majority of SLC35 family members, localized to the Golgi apparatus or the endoplasmic reticulum, SLC35F2 has been reported to localize at the outer cell membrane.^{19–21} In line with this, we assessed if SLC35F2 is facilitating the cellular uptake of YM155 by directly measuring intracellular drug concentration using Multiple Reaction Monitoring, a mass spectrometry-based assay. We observed a ten-fold decrease in intracellular drug levels in KBM7^{GT1} cells as opposed to KBM7^{WT} cells. Moreover, we found that in the reconstituted clone, intracellular drug levels were even higher than in the parental KBM7^{WT} cells, suggesting that SLC35F2 protein levels are proportional to drug uptake (Figure 3a). Indeed, transient overexpression of C-terminal V5 tagged SLC35F2 in HEK293T cells yielded a fifteen-fold increase in intracellular drug levels compared to empty vector control

(Supplementary Figure 5). Hence, the unique reliance of YM155 on the presence or absence of the SLC35F2 gene product may be explained by the role of the protein in determining intracellular YM155 concentrations.

If the levels of SLC35F2 are the main determinant of sensitivity of cells to YM155 action, interference with endogenous expression levels should affect the efficacy of the drug also in other cancer cell lines. The SW480 colon adenocarcinoma cell line features relatively high SLC35F2 mRNA expression levels.²² To validate the importance of SLC35F2 also in this cellular background, we made use of RNA guided Cas9 nuclease-based genome editing technology (CRISPR).^{23,24} We derived two independent isogenic clones SW480^{SLCmut1} and SW480^{SLCmut2} with frameshift mutations in the SLC35F2 gene (exon 7) due to indel mutations (Supplementary Figure 6). These two genomic alterations in SLC35F2 reduced sensitivity of SW480 cells to YM155 more than 100-fold (Figure 3b). This difference in sensitivity was confirmed also in longer-lasting colony formation assays (Figure 3c). To test if SLC35F2 expression levels were not only required but could also further enhance YM155 induced cytotoxicity, we retrovirally overexpressed c-Flag tagged SLC35F2 in A549 lung cancer cells that feature average endogenous SLC35F2 mRNA expression levels.²² Stable overexpression of SLC35F2 in A549 cells resulted in hypersensitivity to YM155 treatment in short- as well as long-term conditions of treatments (Figure 3d, 3e). Collectively, the data suggested that the importance of SLC35F2 in mediating the efficacy of YM155 extended to cancer cell lines more relevant to the clinical implications of YM155 and that in general, the expression levels of this uncharacterized transporter may be the major influx modulating factor.

SLC35F2 expression levels predict YM155 efficacy

Given the fact that ectopic expression of SLC35F2 creates a synthetic hypersensitivity to YM155, we charted SLC35F2 expression levels using a patient-data compendium meta-analysis (OncomineTM). We found that SLC35F2 had been reported overexpressed in a variety of human malignancies compared to the respective non-tumor tissue (Supplementary Figure 7). To test if efficacy of YM155 can be predicted by SLC35F2 expression levels, we derived EC₅₀ values for YM155 as well as for two structurally different DNA damage inducing agents (topotecan and idarubicin) from ten-point dose response curves established in fifteen cell lines with a variable degree of SLC35F2 expression (Figure 4a, 4b, Supplementary Figure 8).²² By comparing YM155 EC₅₀ values to SLC35F2 expression data we found a significant (p-value=0.0007) negative correlation (R=-0.77) over the entire cell line panel tested (Figure 4b). In agreement with our genetically engineered models (KBM7^{GT}, SW480^{SLCmut}), this outlines an enhanced sensitivity to YM155 in cancer cells with endogenously elevated SLC35F2 expression levels. Notably, there was no significant correlation with either of the other agents tested, illustrating the specificity of the YM155-SLC35F2 relationship (Supplementary Figure 8). We next calculated the Pearson's correlation values for all quantified genes and identified SLC35F2 as one of the most significant predictors for YM155 efficacy also on a genome wide scale (Supplementary Figure 9, Supplementary Table 3). Of note, we did not find any significant correlation between YM155 efficacy and survivin expression levels, suggesting that cell lines with elevated survivin levels are not preferentially killed by YM155. We extended our studies

using two members of the cell line panel featuring either high (SW480) or low (HUH7) SLC35F2 expression levels. Also in colony formation assays and long-term drug treatment we could confirm the selective toxicity of YM155 for SLC35F2^{high} cells at natural expression level differences (Figure 4c). To test the hypothesis, that the death-inducing mechanism is indeed DNA damage also in cancer cell lines other than KBM7, and mainly depends on intracellular drug concentrations, we monitored DNA damage in SLC35F2^{high} versus SLC35F2^{low} cells. We treated SW480 cells and HUH7 cells with increasing concentrations of YM155 and probed for canonical DSB markers γ H2AX and phospho-KAP1. A pronounced increase of both markers was observable in SW480 cells, but not in HUH7 cells, indicative of selective DNA damage (Figure 4d, Supplementary Figure 11). We measured intracellular drug levels in both cell lines using targeted mass spectrometry. After treatment with 300 nM or 1 μ M YM155 for 90 minutes, we observed significantly higher levels of YM155 in SW480 cells compared to HUH7 cells (Figure 4e). This confirmed that the reduced transporter expression causes lower intracellular YM155 concentrations, hence causing differential induction of DNA damage. However, an additional contribution to the observed differences caused by differential expression of drug efflux pumps should not be excluded. Altogether, this raised the intriguing possibility that using SLC35F2 levels as discriminating factor, one could predict the efficacy of YM155 also *in vivo*.

SLC35F2 is a major determinant of YM155 efficacy *in vivo*

To test this hypothesis and obtain initial preclinical proof-of-concept for a pharmacological strategy of selective induction of DNA damage we sought a genetically well-defined experimental set-up. We used SW480 cells that were either wildtype for SLC35F2 or had a CRISPR induced frameshift mutation, as described above, to perform a mouse xenograft experiment and drug treatment (Supplementary Figure 6). Cells were injected subcutaneously into flanks of *SCID* mice and YM155 treatment was initiated after tumors reached a volume of $>100\text{mm}^3$. While tumors derived from SW480^{WT} cells rapidly receded, there was no marked decrease of tumor growth with the SW480 clone mutant for SLC35F2 (Figure 4f, Supplementary Figure 10a). Selective induction of a DNA damage response along with induction of apoptosis was assessed via immunohistochemistry using antibodies for γ H2AX and active caspase 3. In line with the *in vitro* results, we found that YM155 treatment caused a selective induction of DNA damage that was dependent on SLC35F2, as it was not observed in tumors derived from SLC35F2-deficient SW480 cells (Figure 4g, Supplementary Figure 10b). Consequently, apoptosis downstream of DNA damage was also only successfully induced in tumors derived from SW480^{WT} cells (Figure 4h, Supplementary Figure 10c).

Collectively, these results indicate that YM155 is capable of selectively inducing DNA damage *in vivo* at clinically relevant drug concentrations.

Discussion

Large scale mutagenesis screens in haploid model organisms like yeast have created insights into fundamental biological processes.²⁵ Here, we have applied global gene-disruption in human haploid cells in order to unravel the cellular consequences of, and genetic

requirements for, exposure to the small molecule drug YM155. Our data suggest that YM155 confers its cytotoxicity via DNA intercalation, causing a DNA damage response that leads to apoptotic cell death. We did not find evidence for a down regulation of survivin on the transcript level, nor did, over the cell line panel we assayed, survivin levels correlate with the efficacy of YM155 to induce cell death. We present evidence for a case where expression levels of only a single, until now uncharacterized, solute carrier gene can determine the efficacy of a clinically evaluated compound. In this case, differential activity of members of the ABC family of transporters responsible for drug efflux does not seem to be determinant. While previous work has established the import of YM155 via OCT1 and OCT2 (SLC22A1 and SLC22A2) as an important pharmacokinetic parameter, we did not observe indication for involvement of these proteins for the uptake of YM155 into the tumour cells assayed. 26,27 However, the residual activity of YM155 on KBM7^{GT1} and KBM7^{GT2} could result from transport occurring by low levels of SLC22A1 and/or SLC22A2 expression.

Systematic charting of target spectra of an increasing number of solute carriers combined with detailed knowledge on their expression patterns in normal physiologic conditions as well as in pathophysiology should enable better predictions of pharmacokinetic and pharmacodynamic parameters for novel small molecule candidates for clinical assessments. So far, we have not been able to identify the natural substrate of SLC35F2. However, a complete understanding of the endogenous cargo-profile of SLC35F2 will be important for understanding the physiological context of differential expression in tissues and tumours.

Collectively, our results indicate that SLC35F2 is an important flux modulator in a way that establishes this carrier as a highly relevant biomarker for further clinical evaluation of YM155. Supported by ectopic overexpression, correlative cell line panel data, genome-engineering loss of function as well as *in vivo* data, we believe that this unique dependency might expand the therapeutic window for YM155 significantly for patients with elevated intratumoral SLC35F2 expression levels. Our data clearly establishes SLC35F2 expression levels as a mechanism-based biomarker stratification strategy for future clinical evaluations of YM155. We believe that the concluded clinical trials with YM155 would have greatly benefitted from this information and that the molecular basis for transport across membranes should be part of the knowledge package accompanying drug candidates. Most importantly, given the overexpression of SLC35F2 in several malignancies, we propose that this gene-drug interaction might be enabling a safe and specific targeting strategy of DNA damage to tumour cells with elevated levels of SLC35F2 expression.

Online Methods

Cell lines and reagents

A549, HUH7, SW480, SW620, HCC38, HCC1187, HS578T, MDA-MB-231, DU4475, MV4-11, HCC2218, BT-549, MCF-7, HCC1419, PC-3 and HEK 293T cell lines were obtained from the American Type Culture Collection and were cultured in the suggested media. YM155, idarubicin-HCl, topotecan-HCl and camptothecin were purchased from Selleck Chemicals (Houston, TX, USA) and nilotinib was purchased from LC Laboratories (Woburn, MA, USA). Aphidicolin, Neocarzinostatin and Ethidium Bromide were purchased

at Sigma Aldrich. Antibodies used in these study: α -Flag (Sigma), α -Actin (Cytoskeleton), α -Kap1, α -phospho Kap1 (both Bethyl Laboratories), α -gH2AX, (Millipore).

Haploid genetic screen and sequence analysis

Haploid genetic screening was performed as described recently.¹³ In brief, virus was produced by transient transfection of the gene-trap plasmid along with packaging plasmids using Lipofectamine 2000 (Invitrogen) in low-passage HEK 293T cells. Virus was concentrated via ultracentrifugation and used to mutagenize 1×10^8 KBM7 cells via spinfection. The mutagenized pool was expanded for another week. Subsequently 1×10^8 gene-trapped cells were selected with 100 nM YM155 in 96 well plates (1×10^5 cells seeded/well). Drug resistant clones were pooled after 10 days of drug exposure, collected in a T175 flask and expanded to a total cell number of 3×10^7 cells. Genomic DNA was isolated and retroviral insertion sites were detected via an inverse PCR protocol adapted to next generation sequencing.¹³ The significance of the enrichment of insertions in a given gene was calculated by comparing the number of insertions of the YM155 selected population with an unselected, larger control dataset using the one-sided Fisher's exact test. The resulting p-values were false-discovery rate corrected using the Benjamini & Hochberg method.

Subcloning of SLC35F2 deficient KBM7 mutant cell line

Due to the high mutational burden of SLC35F2 in the YM155 selected pool (approximately 16% of all mapped insertions clustered in the SLC35F2 gene), no serial subcloning strategy was required. Instead, KBM7^{GT} cells were seeded at a density of 0.1 cells/well in 384 well plates. Monoclonal colonies were then propagated to 96 well plates and DNA was isolated when 96 wells were near confluent. A nested PCR strategy (sequence of primers upon request) was conducted in order to identify clones harbouring a retroviral insertion directly after the first exon of SLC35F2. For final confirmation of gene-trap-localization, the identity of the resulting PCR-product was confirmed by Sanger sequencing.

Determination of intracellular drug levels via multiple reaction monitoring

For YM155, 1×10^6 cells of each assessed genotype (KBM7^{WT}, KBM7^{GT1} and KBM7^{recon}) have been treated with 2 μ M YM155 for 2 hours at 37°C. Subsequently, cells have been washed three times with ice-cold PBS and directly lysed in 300 μ l 80% ice-cold methanol. Lysates were then cleared by centrifugation for 20 minutes at 4°C at 16000 g, supernatants were used for subsequent quantifications by MS. MRM settings were automatically generated using the IntelliStart software (Waters) and quantification was conducted based on the intensity of three daughter ions. Quantification in other cell lines has been conducted using the same setup.

RNA Sequencing

5×10^5 cells (KBM7^{WT} and KBM7^{GT1}) have been treated for six hours with 1 μ M YM155 in biological duplicates. RNA has been isolated using RNeasy kit (Qiagen) and library preparation has been performed with TrueSeq RNA v2 kit (Illumina). Reads were aligned to human reference genome build hg19 using TopHat and Bowtie2.^{28,29} RPKM values were

computed using RSeQC package.³⁰ Fold changes were calculated using R/DEGseq package applying the MARS (MA-plot-based method with Random Sampling model) method at an FDR = 0.001.^{30,31}

Intercalation assay

DNA intercalation assay was performed as described elsewhere.¹⁶

Viability assays

Cellular Viability was assayed using the Cell titer glo assay (Promega) according to the manufacturer's recommendations. Standard assay setup consists of 72 hours drug exposure at various concentrations with 10000 cells seeded initially in 96 well plates. EC₅₀ determination and curve fitting was conducted using Prism software (GraphPad Software).

Apoptosis assays

Induction of apoptosis was determined sixteen hours post drug exposure using the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen™) following the manufacturer's recommendations.

CRISPR targeting strategy

CRISPR targeting sites have been extracted from genome browser tracks downloaded at http://www.genome-engineering.org/crispr/?page_id=41.²³ The following oligos for sense (s) and antisense (as) have been used to generate targeting constructs by cloning into the pX330 backbone as described elsewhere.^{31,32} CACCGAGTGCCACTTCCGTCAACCT (s), AAACAGGTTGACGGAAGTGGCACTC (as). Targeting constructs have been transiently transfected with GFPmax vector (Amara) at a 2:1 ratio. GFP positive cells of the transfected pools have been single cell sorted in 96 well plates and grown and expanded monoclally. For sequence analysis of the targeted locus in SLC35F2 Exon 7, the following primers have been used for PCR amplification forward: ACATCCACGTGGCAAGCACT, reverse: GGCCATACCATCGAAGATGA. 8 PCR products each for SW480^{WT}, SW480^{SLCmut1} and SW480^{SLCmut2} have been subcloned using TOPO blunt end cloning kit (Invitrogen) and sequenced using M13 forward and reverse standard primers.

Xenograft experiments

SW480 cells (2*10⁶ cells) were injected into the flanks of six-week-old *SCID* mice and allowed to reach a tumor volume of >100 mm³ (length × width² × 0.5). Mice were randomized into groups (n = 6) to receive s.c. saline control or YM155 (10 mg/kg*day) administered as a 7-day continuous infusion using an implanted microosmotic pump (Alzet model 1007D, Durect). YM155 was dissolved and diluted in saline immediately prior to administration. Statistically significant differences were determined using Student's t test. P < 0.05 was chosen as the threshold for statistical significance. Mice were housed in the pathogen-free animal facility of the Spanish National Cancer Research Centre (CNIO, Madrid) following the animal care standards of the institution. All animal protocols were approved by the ISCIII committee (Madrid) for animal care and research.

Immunohistochemical studies

Tumor samples were fixed with 4% paraformaldehyde and embedded in paraffin. Subsequently, 5-um sections were prepared and stained with either phospho-histone H2AX (Ser139, JBW301, Millipore) or active caspase-3 (R&D Systems) specific antibodies.

High-Throughput Microscopy

High Throughput Mediated analysis of the DNA damage response were performed as previously described.³³ Briefly, SW480 cells were grown on μ CLEAR bottom 96-well plates (Greiner Bio-One). Treatments were added to the culture media at the following concentrations: 0.1uM YM155, 5uM aphidicolin and 50ng/mL Neocarzinostatin. EdU was added to the media the last 45 minutes of incubation with the drug. EdU incorporation into DNA was detected using the Click-iT™ EdU Alexa Fluor® Imaging kit (Invitrogen/Molecular Probes, Eugene, OR). All steps of the Click-iT™ reaction were performed at room temperature. γ H2AX immunofluorescence was performed using standard procedures. γ H2AX (Upstate Biotechnology) as well as secondary antibody conjugated with Alexa 488 (Molecular probes) were used. Images from each well were automatically acquired by an Opera High-Content Screening System (Perkin Elmer) at nonsaturating conditions with a 20 $\times$ magnification lens. Images were segmented using the DAPI staining to generate masks matching cell nuclei from which the mean signals were calculated. Data were represented with the Prism software (GraphPad Software).

Comet assay

SW480 cells were grown on six-well plates. 100nM YM155 was added to the culture media for 3h. For the irradiation condition, 20Gy were used. Comet assays were performed with the Comet Assay Kit (Trevigen) following the manufacturer's instructions. Images were acquired with a confocal microscope. Mean tail moments (tail length $\times$ tail DNA %) were automatically scored using the OpenComet analyzing software and data were represented with the Prism software (GraphPad Software).³⁴

Statistics

Two-tailed *t* tests were used for statistical analysis if not stated otherwise in the respective figure legends or online methods. A difference was considered significant when $P < 0.05$ (*, *** represents a $P < 0.0005$).

Raw sequencing data

Raw data of the haploid genetic screen is available from the European Nucleotide Archive under the accession number PRJEB6449. RNA sequencing data is available for EBI-ArrayExpress under the accession number E-MTAB-2627.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Mutagenesis of the SLC35F2 locus confers resistance to YM155

(a) The chemical structure of YM155. (b) Circos Plot depiction of sequencing results. Every gene that has been mapped with at least one insertion is depicted as a circle, the localization of which is based on its chromosomal position. The size of a circle correlates with the number of insertions that were mapped for that respective gene. P-values decrease from in-to outside and are depicted as $\log_{10}(-\log_{10})$ values. (c) All mapped insertions in SLC35F2. Red triangles mark insertions that are in sense orientation compared to SLC35F2, blue triangles mark antisense orientations. (d) SLC35F2 locus disruption PCR of KBM7^{WT},

KBM7^{GT1}, KBM7^{GT2}. (e) SLC35F2 and GAPDH mRNA levels in KBM7^{WT}, KBM7^{GT1}, KBM7^{GT2}. (d) and (e) are representatives of three independent experiments. For uncropped gel images of (d) and (e) see Supplementary Figure 11. (f) Cellular viability of KBM7^{WT}, KBM7^{GT1}, KBM7^{GT2} and KBM7^{recon} after 72 hours treatment with various concentrations of YM155. Results represent the mean $\pm$ s.d. of triplicates.

Figure 2. YM155 induces DNA damage selectively in replicating cells expressing SLC35F2
(a) Venn-diagram depiction quantifying number of up- and down regulated transcripts after YM155 treatment in KBM7^{WT} and KBM7^{GT1} cells **(b)** Heatmap depicting the 25 most up- and 25 most downregulated genes after treatment of KBM7^{WT} cells with 1 μ M YM155 for six hours and comparing to the respective alterations in KBM7^{GT1} cells after the same treatment conditions (row normalized). **(c)** Immunoblot analysis of total KAP1, phospho-KAP1 and γ H2AX compared to actin loading control. KBM7^{WT}, SLC35F2^{GT1} and SLC35F2^{recon} cells were treated with the indicated concentrations of YM155 for 24 hours and total cell lysates were used for immunoblotting. Representative of three independent experiments **(d)** DNA intercalation assay. Agarose gel after incubation of relaxed or supercoiled plasmid DNA with different concentrations of topoisomerase (5, 2, 1 and 0.5 Units) in the presence of vehicle control or indicated compounds (camptothecin: 100 μ M, YM155: 100 μ M, EtBr: 10 μ M). (I) and (II) depict the interpretation of the possible results schematically. Representative of two independent experiments. For uncropped gel images of (c) and (d) see Supplementary Figure 11. **(e)** Induction of apoptosis displayed as bargraphs depicting fold-increase in Annexin V positive cells after treatment of KBM7-WT, SLC35F2^{GT1} and SLC35F2^{recon} with indicated concentrations of YM155. Results represent the mean $\pm$ s.d. of triplicates and are normalized to DMSO control of each cell type.

Figure 3. Validation of the dependency of YM155 on SLC35F2 using genome editing

(a) Intracellular YM155 levels as determined by multiple reaction monitoring (MRM) in KBM7^{WT}, SLC35F2^{GT1} and SLC35F2^{recon} cells exposed to 2 μ M YM155 for 90 minutes. Results represent the mean $\pm$ s.d. of triplicates **(b)** Cellular viability of SW480^{WT}, SW480^{SLCmut1} and SW480^{SLCmut2} after 72 hours treatment with various concentrations of YM155. Results represent the mean $\pm$ s.d. of triplicates **(c)** Ten day colony formation assays of SW480^{WT}, SW480^{SLCmut1} and SW480^{SLCmut2} with either vehicle (DMSO) control or indicated concentrations of YM155. Representative of three independent experiments **(d)** Cellular viability of A549 (empty vector control) or A549 cells stably overexpressing C-Flag tagged SLC35F2 after 72 hours treatment with various concentrations of YM155. Results represent the mean $\pm$ s.d. of triplicates **(e)** Ten day colony formation assays of A549 (empty vector control) cells or A549 cells stably overexpressing C-Flag tagged SLC35F2. Representative of three independent experiments.

Figure 4. SLC35F2 is a predictive marker for YM155 efficacy *in vitro* and *in vivo*

(a) SLC35F2 (log₂) expression values of a large set of cancer cell lines reported elsewhere. 22 Cell lines with variable degree of SLC35F2 expression used in correlative study are annotated (b) EC₅₀ values for YM155 were derived for all cell lines indicated and are depicted as a function of SLC35F2 expression levels in the respective cell lines. YM155 efficacy was significantly ($p\text{-value}=0.0007$) negatively correlated with SLC35F2 expression levels ($R=-0.77$). (c) Ten day colony formation assays of SW480 or HUH7 cells treated with either vehicle (DMSO) or the indicated YM155 concentrations. Representative of three

independent experiments **(d)** Immunoblot analysis of phospho-KAP1 and γ H2AX compared to total KAP1 and actin loading controls in SW480 and HUH7 cells treated with the indicated concentrations of YM155 for 24 hours. Representative of three independent experiments. For uncropped gel images see Supplementary Figure 11. **(e)** Intracellular YM155 levels as determined by multiple reaction monitoring in SW480 and HUH7 cells exposed to 0.3 and 1 μ M YM155 for 90 minutes. Results represent the mean $\pm$ s.d. of triplicates **(f)** Tumor size of SW480^{WT} and SW480^{SLCmut2} xenografts after treatment with YM155 (10mg/kg*d) (n=6). Infusion pumps filled with vehicle or YM155 were implanted on animals when tumors reached 100 mm³ and monitored for growth for 1 week. N.S.: non significant; ***: $P < 0,001$ **(g)** and **(h)** Quantification of γ H2AX and C3A positive cells. Results represent the mean $\pm$ s.d. of triplicate analysis