Optimized PAR2 assay using HT-29 PAR1 KO cells generated by CRISPR

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Abstract

Protease-activated receptor 2 (PAR2) is an attractive target for the treatment of inflammation and cancer[1]. But screening and characterization of PAR2-targeting compounds is challenging, since most (if not all) cells also express PAR1. HT-29 cells are commonly used for studying PAR2 since they mainly express PAR2 and only little PAR1[2] – but they still respond to PAR1 agonists. Moreover, PAR1/PAR2 heterodimerization[1] might alter the pharmacology of a given PAR2-targeting compound. Here we used CRISPR to completely knock out PAR1 in HT-29 cells, thereby generating a perfect new cell model for studying PAR2 without any possible interference from PAR1. Using functional Ca^{2+} assays we confirm that HT-29 WT cells do not discriminate between PAR1 vs. PAR2 agonists, while our new HT-29 PAR1 knock out (KO) cells respond only to PAR2, but not to PAR1 agonists any more. For further characterization, due to poor commercial availability of PAR2 antagonists we then first synthesized the described PAR2 antagonist AZ8838[3], and then tested different PAR1 vs. PAR2 antagonists against different PAR1 vs. PAR2 agonist on our HT-29 WT vs. PAR1 KO cells. Most remarkably, against a mixed PAR1/2 agonist, PAR2 antagonists displayed only partial inhibition in the HT-29 WT, but full inhibition in our PAR1 KO cells, confirming again the successfull knock-out.

Generation of HT-29 PAR1 KO Cells Using CRISPR

Cells	Genotypes	DNA Sequences (Exon 2)	Amino acid sequences
HT-29 WT	Homo-zygous wild-type	AGCAACAAATGCCACCTTAGATCCCCGGTCATTTCTTCTCAGGAACCCCAATGATA (gRNA underlined)	MGPRRLLLVAACFSLCGPLLSARTRARRPESKATNATLDPRSFLLRNP NDKYEPFWEDEEKNESGLTEYRLVSINKSSPLQKQLPAFISEDASGYL TSSWLTLFVPSVYTGVFVVSLPLNIMAIVVFILKMKVKKPAVVYMLHL ATADVLFVSVLPFKISYYFSGSDWQFGSELCRFVTAAFYCNMYASILL MTVISIDRFLAVVYPMQSLSWRTLGRASFTCLAIWALAIAGVVPLLLK EQTIQVPGLNITTCHDVLNETLLEGYYAYYFSAFSAVFFFVPLIISTV CYVSIIRCLSSSAVANRSKKSRALFLSAAVFCIFIICFGPTNVLLIAH YSFLSHTSTTEAAYFAYLLCVCVSSISCCIDPLIYYYASSECQRYVYS ILCCKESSDPSSYNSSGQLMASKMDTCSSNLNNSIYKKLLT (425)
HT-29 PAR1 KO Clone #48	Compound hetero-zygous mutant	1st allele: AGCAACAAATGCCA--------CCCCGGTCATTTCTTCTCAGGAACCCCAATGATA (8 bp deletion, resulting in additional 9 new amino acids and a premature stop codon) 2nd allele: AGCAACAAATGCCACCTT----------CTCAGGAACCCCAATGATA (19 bp deletion, resulting in additional 22 new amino acids and a premature stop codon)	From 1st allele: MGPRRLLLVAACFSLCGPLLSARTRARRPESKATNATPVISSQEPQ (46) From 2nd allele: MGPRRLLLVAACFSLCGPLLSARTRARRPESKATNATFSGTPMINMNH FGRMRRKMKVG (59)

Figure 1. Generation of HT-29 PAR1 KO cells. Guide RNAs (gRNAs) targeting 3 different sequences in the PAR1 gene were designed. HT-29 cells were electroporated with a ribonucleoprotein (RNP) complex of the gRNAs with Cas9 nuclease. After ~ 1 week, the genomic DNA of the targeted sites was sequenced in order to verify the PAR1 KO in the 3 cell pools. The pool displaying the highest KO rate was selected for limiting dilution to generate individual clones. Then genomic DNA of the targeted exon was sequenced again in several individual clones. Clones harboring frameshift mutations were selected for further amplification. Finally, the HT-29 PAR1 KO cell clones (vs. HT-29 WT cells) were tested in a functional Ca^{2+} assay to confirm the loss of activation by the PAR1-selective TFFLR-NH_2 (see also Figure 3.). The table depicts the PAR1 DNA and protein sequences of the finally selected PAR1 KO cell clone as compared to the HT-29 WT cells.

Synthesis of the PAR2 Antagonist AZ8838

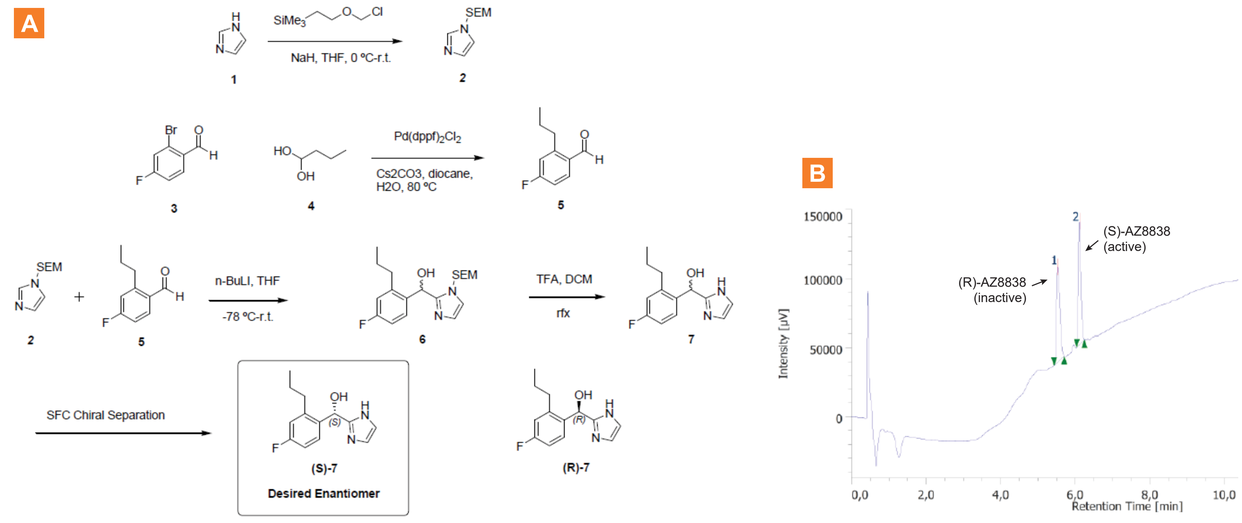

Figure 2. Synthesis of the described PAR2 antagonist AZ8838[3] (not commercially available) as an addition to the commercially available PAR2 antagonist I-191[2]. A. Multi-step synthesis of AZ8838. B. Chromatographic separation of (S)- from (R)-AZ8838.

Agonist Pharmacology of HT-29 WT vs. PAR1 KO Cells

HT-29 WT vs. PAR1 KO cells were stimulated with different PAR1 vs. PAR2 agonist proteases and peptides, respectively. As expected, the HT-29 WT cells respond to both, PAR1 and PAR2 agonist, while the PAR1 KO cells respond only to PAR2, but not to PAR1 agonists any more.

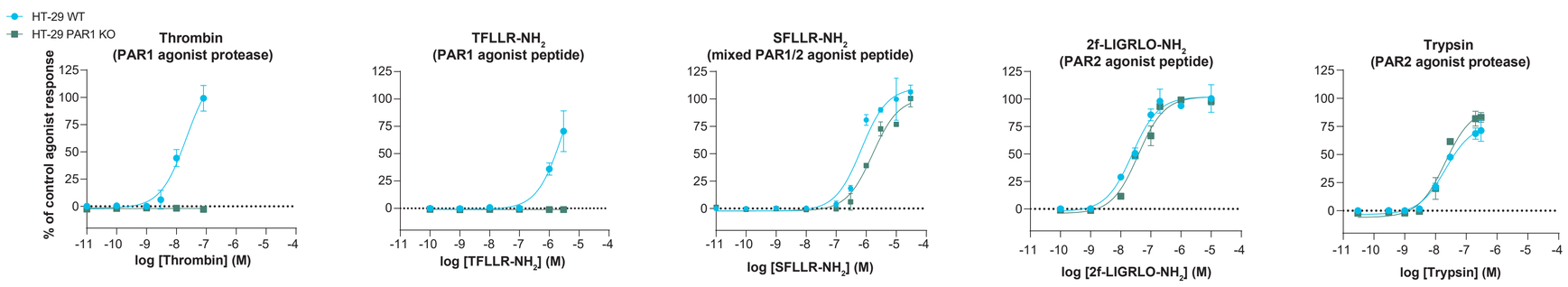

Figure 3. PAR1 / PAR2 agonist assays in HT-29 WT vs. PAR1 KO cells. Cells were loaded with the Ca^{2+}-sensitive dye Fluo-4, then different PAR1 / PAR2 agonists were added, and the agonist-induced Ca^{2+} signals were measured. For each cell line, the signals obtained with the different agonists are normalized with respect to the maximal signal obtained with the PAR2-selective peptide 2f-LIGRLO-NH_2.

Antagonist Pharmacology of HT-29 WT vs. PAR1 KO Cells

HT-29 WT vs. PAR1 KO cells were pre-incubated with different PAR1 vs. PAR2 antagonists, then stimulated with the different agonist proteases and peptides selective or not for PAR1 vs. PAR2, respectively.

As expected, in HT-29 WT cells (expressing both, PAR1 and PAR2), the PAR1 antagonist Vorapaxar had no effect against the PAR2-selective agonists, was a full antagonist against the PAR1-selective agonists, and was a weak partial antagonist against the mixed PAR1/2 agonist; and this latter effect disappeared in the PAR1 KO cells.

Likewise, in HT-29 WT cells, the PAR2 antagonists I-191 and AZ8838 had no effect against the PAR1-selective agonists, were full antagonist against the PAR2-selective agonists, and displayed partial antagonism against the mixed PAR1/2 agonist; but this latter effect turned into full antagonism in the PAR1 KO cells.

These partial effects in the HT-29 WT cells disappearing or turning into full inhibition, respectively, in the KO cells again nicely confirm the PAR1 knock-out.

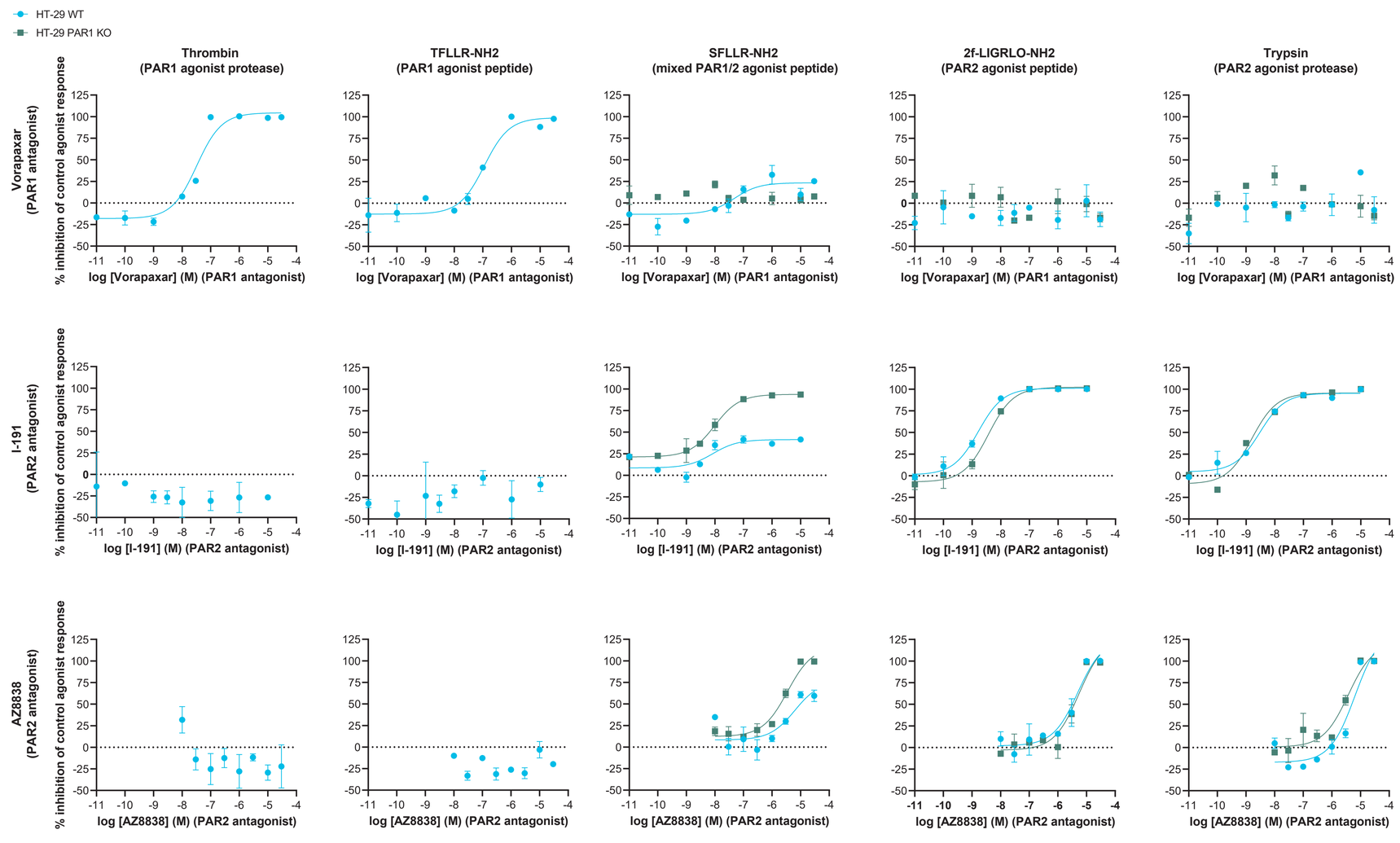

Figure 4. Antagonist assays. As in the agonist assay (Figure 3.), except that the cells were pre-incubated (5 minutes) with the different antagonists before addition of the different agonists, each at its EC_{80}. The signals are normalized with respect to the corresponding signals obtained without antagonists.

Summary

- Using CRISPR we generated an HT-29 PAR1 KO cell line. We then characterized these cells in *in vitro* pharmacological assays using different PAR1 vs. PAR2 angonists and antagonist, including the commercially unavailable AZ8838 which we synthesized specifically for this study. Our results nicely confirm that our new HT-29 PAR1 KO cells now permit to study PAR2 without any interference from PAR1, and are therefore a highly valuable tool that greatly facilitates screening and characterization of any PAR2-targeting compounds.
- Accordingly, we will now replace our previous PAR2 assay using a desensitization step to avoid interference from PAR1 by our new PAR2 assay on our new HT-29 PAR1 KO cells (ref. G372 / 4709 / 4710) which can be used directly without the additional PAR1 desensitization.
- More generally, this case study on PAR2 nicely demonstrates how our integrated capacities comprising CRISPR cell line generation, chemical compound synthesis, and *in vitro* pharmacology assay development can be applied for generating optimal tools and assays for any challenging compound testing / screening project also on any other pharmacological target.

References

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2. Jiang Y, *et. al.* A potent antagonist of protease-activated receptor 2 that inhibits multiple signaling functions in human cancer cells. *The Journal of Pharmacology and Experimental Toxicology* 2018; 364:246-257.
3. Cheng RKY, *et al.* Structrural insight into allosteric modulation of protease-activated receptor 2. *Nature* 2017; 545:112-115.