Molecular Pharmacology

Supplemental Material

Pore exposed tyrosine residues of P-glycoprotein are important hydrogen bonding partners for drugs

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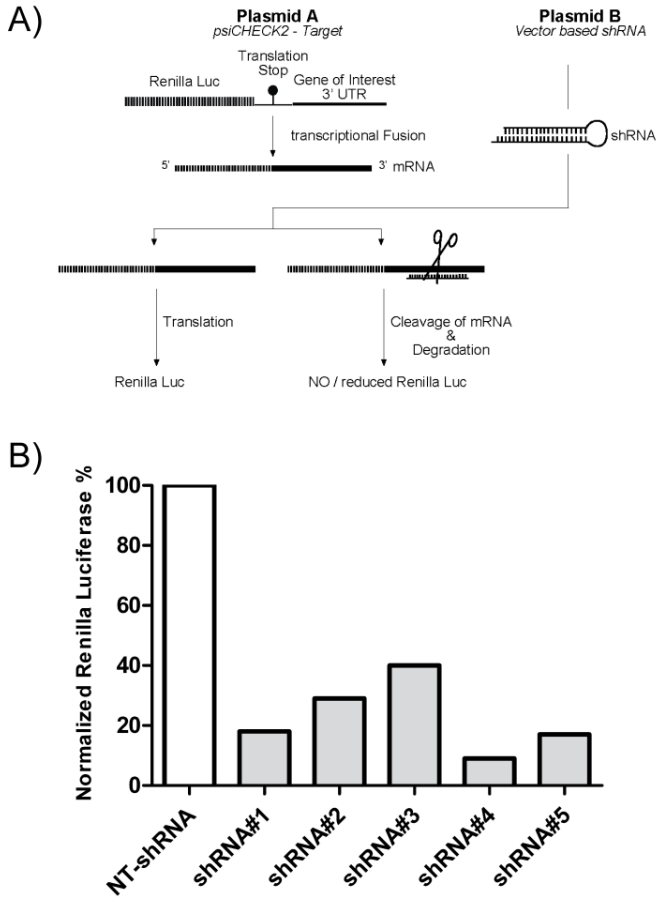
Supplementary Figure 1

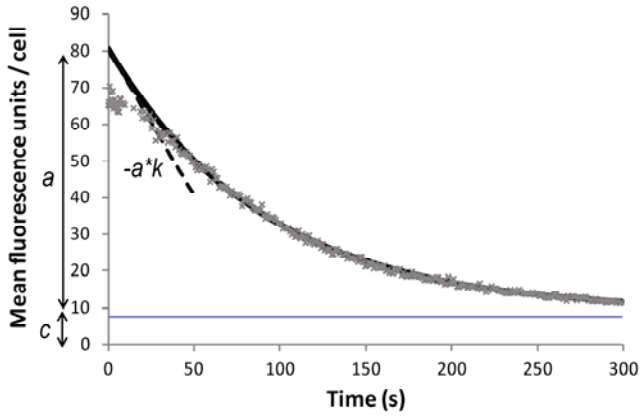


A. Mechanism of action of the siCHECK-2 Vector Reporter System. Plasmid A: The target region (3'UTR of endogenously expressed P-gp) is cloned into the multiple cloning region located 3' to the synthetic Renilla gene and its translational stop codon. Plasmid B: Vector producing short hairpin (sh) RNA. Reporter (Plasmid A) and vector based shRNA constructs

(Plasmid B) are co-transfected into HEK293 cells. A fusion of the Renilla gene and the gene of interest is transcribed. Effective shRNA constructs will initiate the RNAi process towards the target gene. The fused Renilla-gene of interest mRNA will be cleaved and subsequently degraded, decreasing the Renilla luciferase signal.

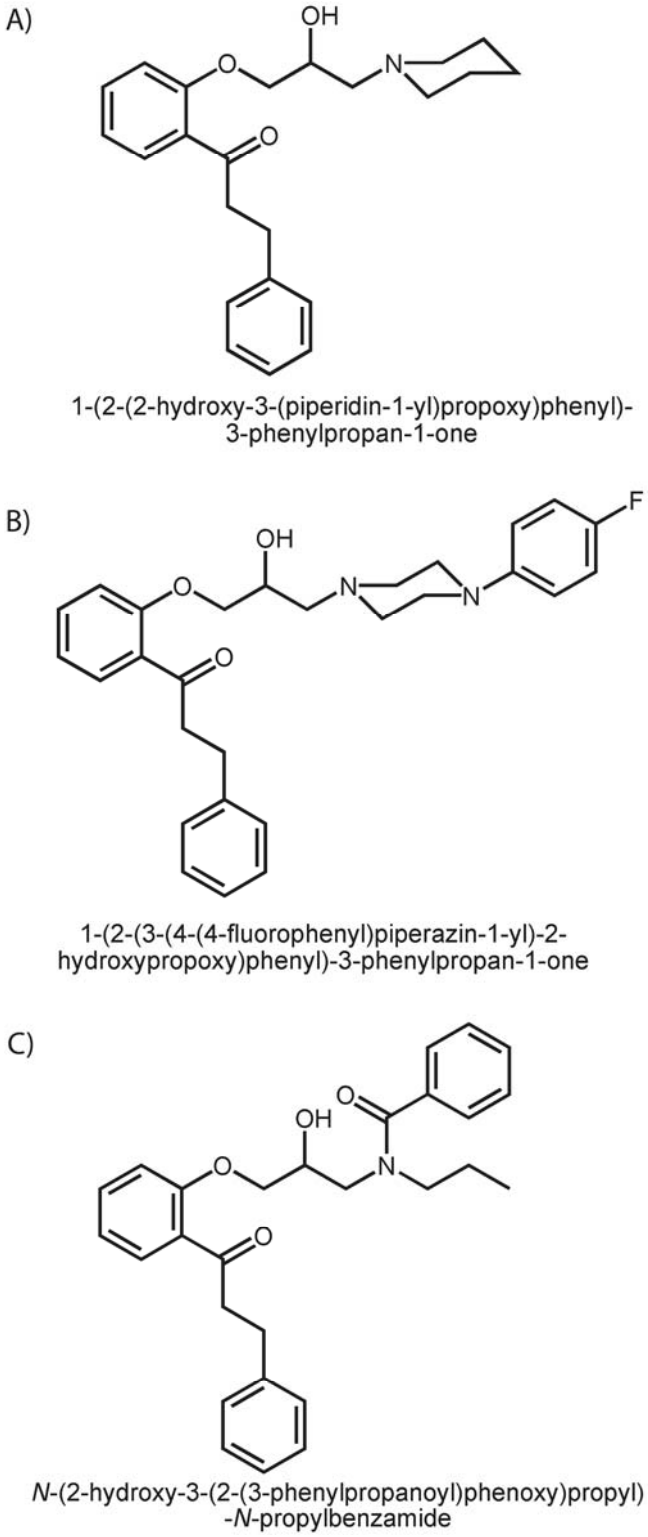
B. Identification of effective siRNAs targeting the 3 'UTR of endogenous expressed P-gp. A 384 bp fragment corresponding to the 3' UTR region was cloned into the psiCheck 2 Vector, using the Xho I and Not I restriction sites located 3' to the Renilla translational stop codon. Five constructs (#1 - #5) expressing the potential shRNAs were co-transfected into HEK293 cells and analyzed for target mRNA degradation. NT-shRNA is a control vector containing scrambled shRNA (Addgene plasmid # 1864). Constructs #1 and #4 showed most potent target downregulation. Renilla and Firefly luciferase activities were measured 48 hours post transfection using the Dual-Glo Luciferase Assay System (Promega). Renilla luciferase has been normalized to firefly luciferase. Values represent the average of triplicate independent experiments, with the ranges indicated. All shRNA expression cassettes were sequence verified.

Supplementary Figure 2



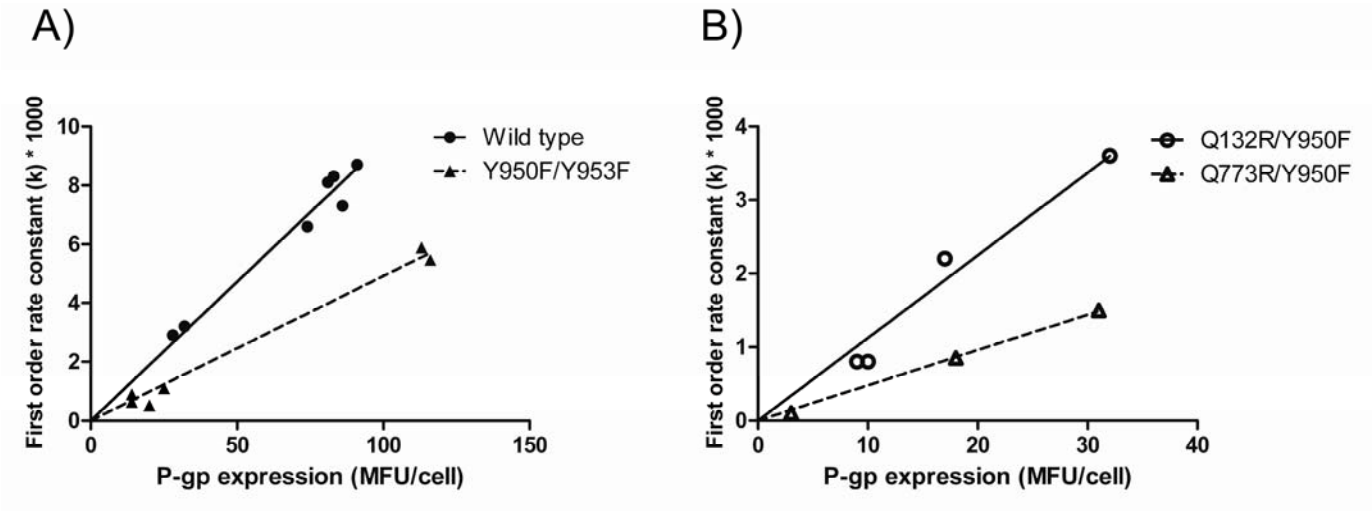
Rh123 efflux was measured continuously over five minutes and mean fluorescence units per cell were determined from about 3000 gated events every second. An exponential curve was fitted to 300 individual data points (x), each representing the average over one second. During the first 30 seconds (resuspension of cells in warm efflux medium) the transport rate is lower, because cells have to readapt from being shifted from ice to 37°C. These data points were therefore excluded from the fit. First order rate constants (*k*-values) were calculated from an exponential fit (solid line) according to the equation $y=a*e^{-kt}+c$, where *a* is the difference between zero and infinite time point of the curve, *e* is the Euler number, *k* is the first order rate constant, *t* is the time in seconds and *c* is the background fluorescence of cells. The slope of the tangent in the origin (stippled line) is *-a*k*. It is important to note that the variable k is thus independent of initial loading, because *-a*k/a* is equal to *-k*. Therefore *-k* can be viewed as a tangent in the origin of the curve for data points referring to fractional cellular fluorescence (normalized initial efflux rate). There is no need for attempting to load cells equally by e.g. depleting them of ATP or inhibiting efflux transport during loading. Both re-establishing cellular energy metabolism after ATP depletion or washing out inhibitors before functional assays could potentially compromise results because of the required time scales.

Supplementary Figure 3



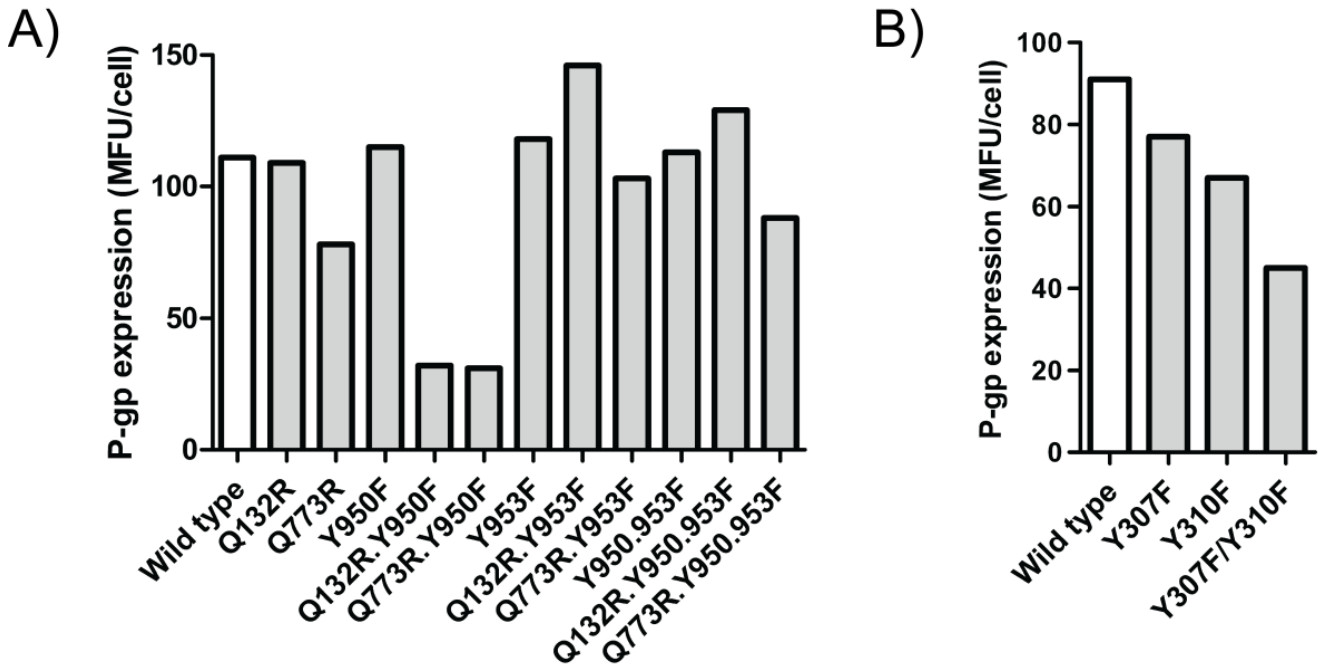
Chemical structures of protonatable propafenones GPV005 **(A)**, GPV031 **(B)** and the non-protonatable acid amide GPV366 **(C)**.

Supplementary Figure 4



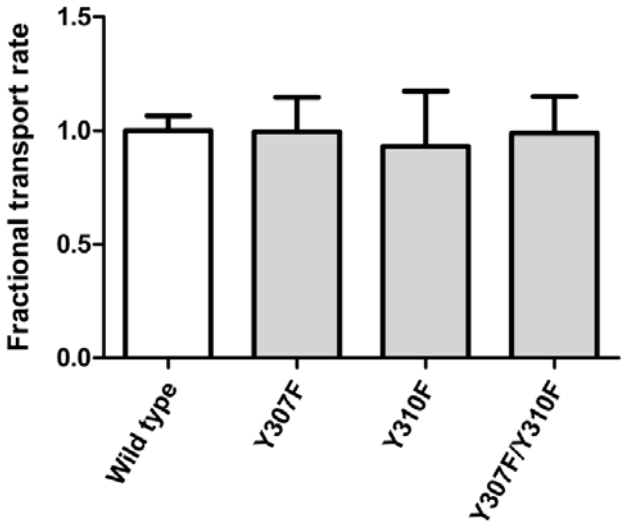
First order rate constants are plotted as a function of P-gp expression in order to show the linear relationship between first order rate constants and expression. Transport measurements were done in duplicates and expression measurements were done in triplicates for each experiment. Mean values are shown for wild type P-gp and the Y950F/Y953F mutant **(A)** and the Q132R/Y950F and Q773R/Y950F mutants **(B)**. The latter show lower surface expression.

Supplementary Figure 5



Expression levels of wild type P-gp and tyrosine mutations located in helix 11 **(A)** and helix 5 **(B)**. Data are shown for one representative experiment performed in triplicates. P-gp specific MRK16 antibody was used to determine expression levels and IgG2A was used as the control antibody to correct for non-specific binding.

Supplementary Figure 6



Bar graph showing fractional transport rates of Y307F, Y310F and Y307F/Y310F mutants in comparison with wild type P-gp. First order rate constants (k) for rh123 efflux were calculated from an exponential fit and normalized to surface expression determined by MRK16 staining. Each value represents the mean ± SD of at least 3 independent experiments. No significant difference in transport rates was found in mutants as compared with wild type P-gp.

Supplementary Table 1

	IC_{50} ± SD (nM)		
	GPV005	**GPV031**	**GPV366**
Wild type	518 ± 141	85 ± 22	2239 ±391
Y950F	1348 ± 229	238 ± 84	2984 ± 79
Y953F	1207 ± 362	287 ± 109	15946 ± 2941
Y950F/Y953F	2418 ± 194	630 ± 87	17819 ± 2106
Q132R	1414 ± 215	521 ± 183	1074 ± 282
Q132R/Y950F	1360 ± 387	363 ± 127	966 ± 259
Q132R/Y953F	2164 ± 252	1100 ± 118	3261 ± 965
Q132R/Y950F/Y953F	3367 ± 662	1414 ± 324	5286 ± 1465

IC_{50} values of compounds in wild type and mutants, calculated from concentration response curves by non-linear regression analysis. Each value represents the mean ± SD of at least 3 independent experiments.

Supplementary Table 2
A)

	Significance		
	GPV005	**GPV031**	**GPV366**
Wild type vs. Y950F	***	*	ns
Wild type vs. Y953F	**	**	***
Wild type vs. Y950F/Y953F	***	***	***
Y950F vs. Y953F	ns	ns	***
Y950F vs. Y950F/Y953F	***	***	***
Y953F vs. Y950F/Y953F	***	***	ns

B)

	Significance		
	GPV005	**GPV031**	**GPV366**
Q132R vs. Q132R/Y950F	ns	ns	ns
Q132R vs. Q132R/Y953F	ns	**	*
Q132R vs. Q132R/Y950F/Y953F	***	***	***
Q132R/Y950F vs. Q132R/Y953F	*	***	*
Q132R/Y950F vs. Q132R/Y950F/Y953F	***	***	***
Q132R/Y953F vs. Q132R/Y950F/Y953F	**	ns	*

Statistical significance of differences in IC_{50} values for compounds GPV005, GPV031 and GPV366 in wild type **(A)** and Q132R background **(B)**. *p< 0.05, **p< 0.01 ***p< 0.001