Supporting Information

Comparative Ecotoxicological Hazard Assessment of Beta-Blockers and their Human Metabolites using a Mode-of-action Based Test Battery and a QSAR Approach

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Experimental Section

Chemical analysis. The concentrations of the ß-blockers were quantified with HPLC (Summit HPLC System; Dionex, Olten, Switzerland) and UV (UVD 340-U, Dionex, Olten, Switzerland) or fluorescence detection (RF 1002, Gynkotek, Olten, Switzerland). A reversed phase C18 column (125/4 Nucleodur Gravity 5m, Macherey-Nagel, Oensingen, Switzerland) was used for separation. The eluent was composed of buffer (10mM ortho-phosphoric acid and 2 mM 1-octanesulfonic acid at pH 5) and acetonitrile. The buffer-to-acetonitril ratio was 85:15 for atenolol and 60:40 for metoprolol and propranolol. All compounds were detected at 227 nm in the UV detector and the following wavelengths for fluorescence detection: extinction 227 nm, emission 305 nm for atenolol and metoprolol; extinction 224 nm, emission 305 nm for propranolol (1).

Liposome-water partitioning. Small unilamellar liposomes were made from synthetic 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine by the membrane extrusion method (2). Sorption isotherms were determined at a liposome-to-water ratio of 0.8 g_{lip}/L (propranolol), 4 to 8 g_{lip}/L (metoprolol) or 16 to 20 g_{lip}/L (atenolol). Concentrations in the aqueous phase c_w were determined by the HPLC method described above. The concentrations in the liposomes, c_{lip} ($mol \cdot kg_{lip}$) were computed by difference of the aqueous concentrations in the reference dialysis cells without liposomes and the aqueous concentrations in the dialysis cells with liposomes considering the liposome-to-water ratio (3). The D_{lipw}(pH 7) is defined as the ratio of the concentration in the liposomes, c_{lip} and the aqueous concentration c_w. It is given in units of $L \cdot kg^{-1}$ but since the density of the lipid phase is close to 1 $kg_{lip} \cdot L^{-1}$ (3), it can be set equal to a dimensionless partition coefficient for QSAR applications.

Toxicity of ß-blockers in the 30-min bioluminescence inhibition test with the marine bacterium *Vibrio fischeri*

EC50 in the bioluminescence inhibition test were compared with a baseline QSAR for this test system. Since the ß-blockers are positively charged at pH 7, a QSAR based on the liposome-water partitioning D_{lipw}(pH7) is needed to predict the activity. Therefore a QSAR based on K_{ow} from the literature (4) was rescaled to D_{lipw}(pH7) according to the method described in (5). The results indicate baseline toxicity of the three ß-blockers for this endpoint because all experimental data (Figure S-1) are within a factor of 4 in relation to the baseline (0.2 >TR >4) and are below the threshold for specific toxicity that is defined as TR≥ 10 (6).

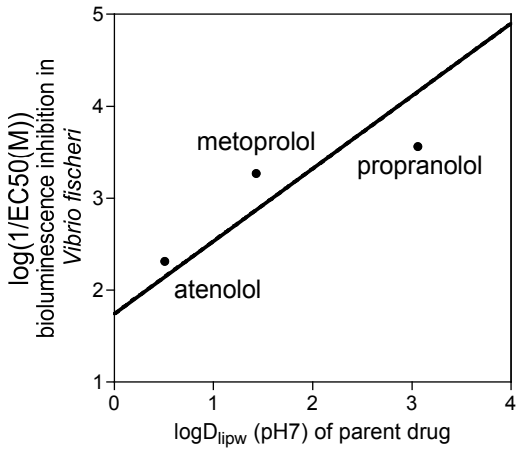

Figure S-1. Baseline QSAR (line) and ● EC50 of the ß-blockers in the bioluminescence inhibition test indicating baseline toxicity.

UmuC test for genotoxicity

Only propranolol showed a significant effect in the umuC test (i.e. induction faction > 1.5 while growth factor > 0.5), but only after metabolic activation with a hepatic S9 extract, and only at millimolar concentrations (Figure S-2).

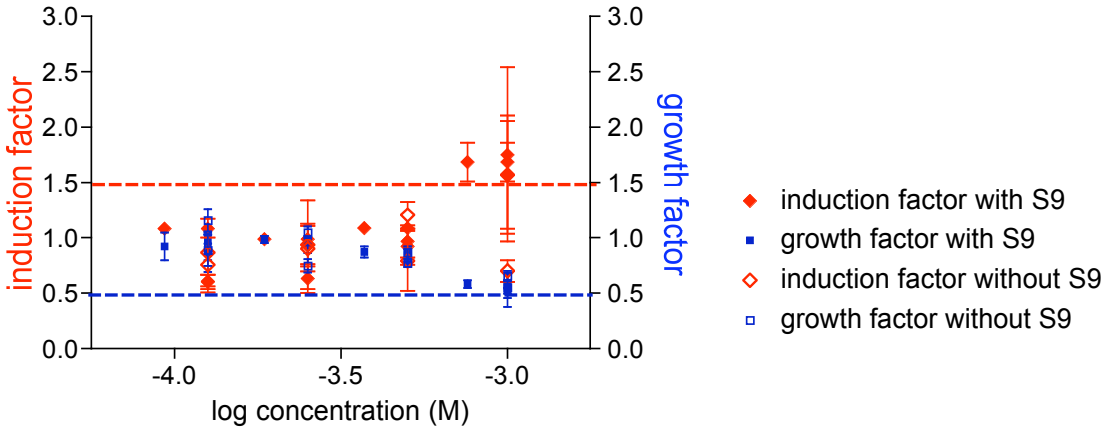

Figure S-2. Effect of propranolol in the umuC test.

Chlorophyll fluorescence test

Figure S-3 depicts the concentration-effect curves of the ß-blockers and the reference compound diuron, from which the EC50 values for inhibition of PSII quantum yield reported in Table 2 of the main paper were derived. The toxicity of the ß-blockers increases with hydrophobicity and they are less toxic than the specific PSII inhibitor diuron but they are all more toxic than predicted from their expected baseline effect. The concentration-effect curves show a relatively high variability. However, the experiments were performed during the course of one year. Overall we measured 42 independent concentration-effect curves for diuron, 14 for atenolol, 11 for metoprolol, and 15 for propranolol. In an attempt to decrease the variability we made many more replications

than initially planned but instead of decreasing the variability we were just able to better describe it.

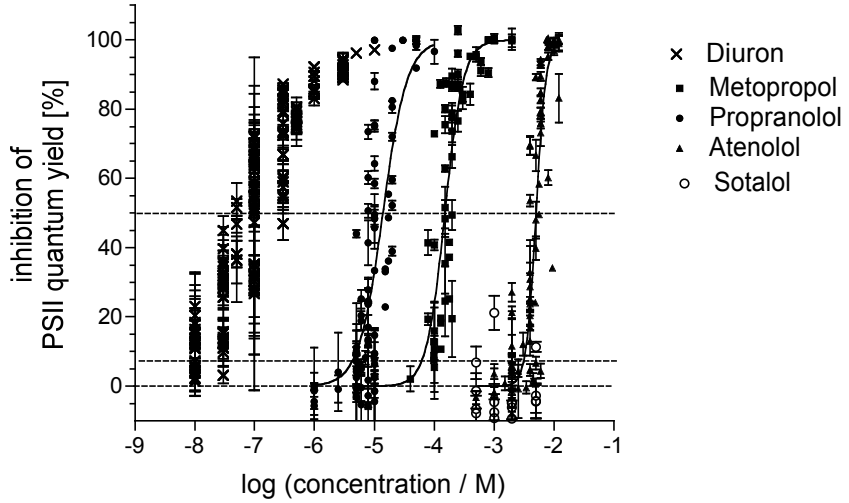

Figure S-3. Concentration-effect curves of the ß-blockers and the reference compound diuron (a specific inhibitor of the photosystem II) in the 24-h chlorophyll fluorescence test with Desmodesmus subspicatus*. The error bars are the 95% confidence intervals.*

In Figure S-4, the concentration-effect curves for the mixture experiments are plotted to support the information of concentration addition given in the main paper.

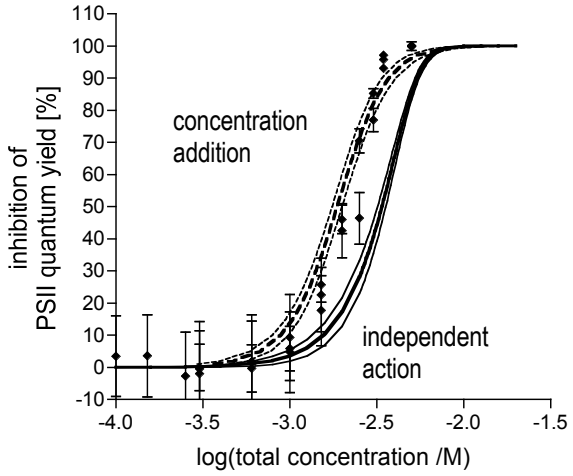

Figure S-4. Concentration-effect curves of the mixtures of three ß-blockers (atenolol, metoprolol, propranolol) in the chlorophyll fluorescence test. The compounds were mixed in the ratio of their EC_{50} values. The diamonds correspond to the experimental data and the error bars are the 95% confidence intervals. The broken lines refer to the prediction for concentration addition; the solid lines refer to the prediction for independent action. For each prediction model, the lower 95% confidence limit is shown on the left, the actual prediction in the middle and the upper 95% confidence limit on the right.

Additional experiments: rapid induction kinetics of chlorophyll fluorescence in dark-adapted algae

The reference compounds, the herbicide diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) caused a dramatic acceleration of fluorescence due to inhibition of the electron transport chain in photosystem II (14). This effect was instantaneous and occurred at concentrations around the EC50 for the chlorophyll fluorescence assay. In contrast, propranolol only showed an effect on the rapid induction kinetics at higher concentrations than in the 24-h test using the ToxyPAM. If 10^{-5} to 10^{-4} M propranolol was added to the algae, the effect on the rapid induction kinetics started immediately, but continued to increase in the next few hours until it became stable after 2 to 3 hours (incubation in dark, no growth). Similar patterns were observed for the other ß-blockers. If the ß-blockers were incubated for 24 hours in light, the effect on photosynthesis was stronger and both measured fluorescence parameters, F and Fm', were quenched. This time dependence of the effect confirms that the ß-blockers interfere indirectly with photosynthesis, because no growth is required to show the indirect effect on photosynthesis.

Comparison of ecotoxicity test results with literature data

The chlorophyll fluorescence assay yielded comparable results as the 72-h growth inhibition assay (7,8). Acute fish toxicity typically occurs at higher concentrations than effects on algae (8,9), but invertebrates are also rather sensitive to ß-blockers. *Ceriodaphnia dubia* appears to be the most sensitive of the tested invertebrate species and exhibits EC50 values in a 48-h mobility test in a similar concentration range as algae (8-10). Since no baseline QSAR is available for these endpoints, it cannot be assessed if this effect is a specific or a baseline toxic effect, but it is clear that ß-blockers do affect the heart rate in *Daphnia* (11). We additionally compared literature ecotoxicity data with baseline QSARs for *Chlorella vulgaris*, *Daphnia magna* and *Pimephales promelas* applied in environmental risk assessment of chemicals (12), which were converted to a lipophilicity descriptor that takes account of the speciation of the ß-blockers (5) (Equations S-1 to S-3). This analysis confirms that the ß-blockers show a specific effect with TR in the range of 20 to 400, while they had TR < 10 for *Daphnia magna* and *Pimephales promelas,* confirming baseline toxicity in the acute toxicity tests for these species like for the bacterium *V. fischeri* investigated in the present study.

Chlorella vulgaris: $\log (1/EC50_{\text{baseline toxicity}}(M)) = 0.91.\ \log D_{lipw}(pH7) + 0.63$ (S-1)

Daphnia magna: $\log (1/EC50_{\text{baseline toxicity}}(M)) = 0.77.\ \log D_{lipw}(pH7) + 1.89$ (S-2)

Pimephales Promelas: $\log (1/LC50_{\text{baseline toxicity}}(M)) = 0.83.\ \log D_{lipw}(pH7) + 1.46$ (S-3)

Table S-1. Analysis of literature data for propranolol

Test species	$\log (1/EC50_{exp}(M))$	$\log(1/EC50_{baseline}(M))$ [a]	TR
Chlorella vulgaris	4.71[b]	3.42	19.3
Daphnia magna	5.03[c]	4.25	6.0
Pimephales promelas	4.09[d]	4.01	1.2

Data from reference [a](5), [b](13), [c](8), and [d](9).

Table S-2. Analysis of literature data for metoprolol

Test species	$\log (1/EC50_{exp}(M))$	$\log (1/EC50_{baseline}(M))$[a]	TR
Chlorella vulgaris	4.53[b]	1.93	397
Daphnia magna	2.79[c]	2.99	0.6
Pimephales promelas	3.43[d]	2.65	6.1

Data from reference [a](5), [b](7), [c](8), and [d](9).

Table S-3. Analysis of literature data for atenolol

Test species	$\log (1/EC50_{exp}(M))$	$\log (1/EC50_{baseline}(M))$	TR
Chlorella vulgaris	2.63[b]	1.19	27.8
Daphnia magna	2.93[c]	2.36	3.7
Pimephales promelas	n.d.	1.97	n.d.

Data from reference [a](5), [b] (7), and [c](8).

Rules for assessing distribution of metabolites

The identity of the human metabolites and the fractions excreted in urine and feces were compiled from literature (15-19) and the Swiss Drug Kompendium (Documed AG, Basel, Switzerland, www.kompendium.ch, last visit to website: 07.11.2005). Since data sets were generally not consistent, we defined ranges of fractions of metabolites with the following rules:

1. In case of conjugation, all conjugates were assumed to be glucoronides. This is a worst-case assumption, because glucoronides, albeit very hydrophilic, constitute the conjugates of relatively highest hydrophobicity.
2. Typically, ranges of fractions of metabolites were given. In addition, data from different sources were often not consistent. Therefore, ranges of fractions were reported with a minimum fraction from summing up the minimum values for single metabolites and a maximum fraction from summing up the maximum values for single metabolites. In a few instances, this procedure resulted in maximum values way above 100%. As a worst-case assumption, we did not re-scale these maximum values. We also did not rescale if the maximum values were below 100 %.
3. If there was only the verbal information in the literature sources: „other metabolites in small amounts“ and there was information on the identity of these metabolites, we set the minimum fraction to 0%, the maximum to 5%.

Reaction schemes of human metabolism of three ß-blockers and product distribution

Propranolol

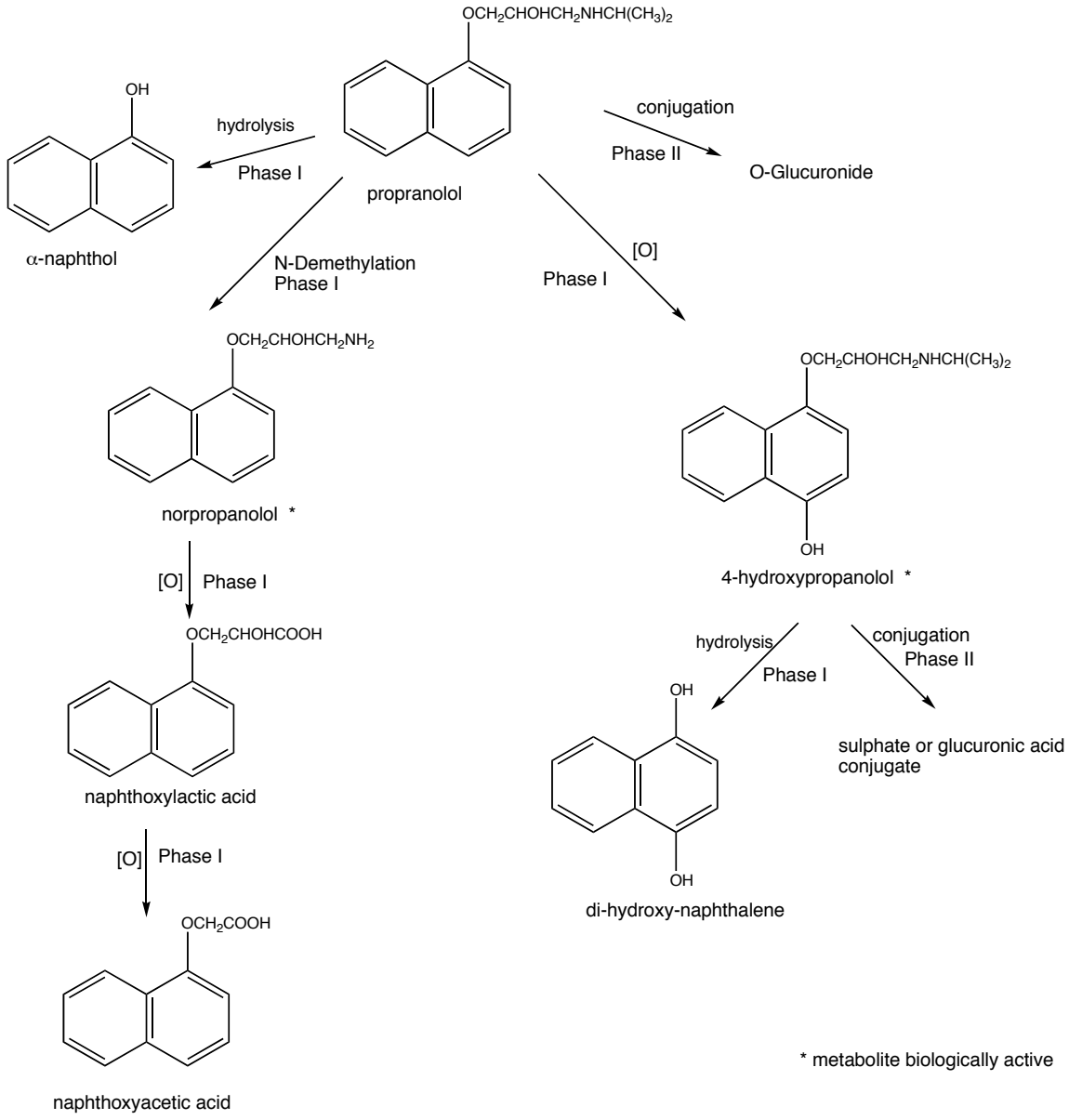

*Figure S-5. Reaction scheme of human metabolism of propranolol. An asterisk * marks those metabolites which are pharmacologically active.*

Table S-4. Product distribution of metabolites in human excretory products and physicochemical descriptors of the metabolites of propranolol.

Metabolite[a]	Fraction in feces (%min to %max)[a]	Fraction in urine (%min to %max)[a]	$\log K_{ow}$	$\log K_{lipw}$ (neutral species)[h]	Speciation at pH 7	$\log D_{lipw}$ (pH7)[i]
Propranolol	0 to 5	0.5 to 4	3.48[b]		base, 99% positively charged	3.07[j]
Propranolol-glucoronide		17 to 25	2.32[c]	2.61	neutral	2.61
4-Hydroxy-propranolol		10 to 41	2.81[d]	3.06	equal to propranolol	2.08
4-Hydroxy-propranolol-glucoronide		0 to 30	1.65[c]	2.01	neutral	2.01
Norpropranolol		0 to 5	2.80[d]	3.05	base, 99% positively charged	2.07
Naphthoxy-lactic acid		22 to 42	1.32[d]	1.71	acid, 99% negatively charged	0.71
Naphthoxy-acetic acid		0 to 20	2.53[e]	2.80	acid, 99% negatively charged	1.80
Naphthol		0 to 5	2.69[f]	2.95	neutral	2.95
Dihydroxy-naphthalene		0 to 5	2.02[g]	2.34	neutral	2.34

[a] Data from ref. (15-19) and Swiss Drug Kompendium (Documed AG, Basel, Switzerland, www.kompendium.ch, last visit to website: 07.11.2005). [b] Data from ref (22). [c] Estimated from K_{ow} of parent compound and K_{ow} for glucoronic acid (-2,57 (23)) [d] Estimated with the fragment method described in (23,24). [e] experimental (23,24). [f] Data from Physprop database , http://www.syrres.com/esc/physprop. [g] Estimated from K_{ow} of naphthol with fragment method (23,24). [h] calculated with eq 4 of the main manuscript $\log K_{lipw} = 0.904 \cdot \log K_{ow} + 0.515$ (25), [i] Corrected for the speciation, assuming $\log D_{lipw}(pH7) = \log(f_{neutral} \cdot K_{lipw} + f_{charged} * 0.1 \cdot K_{lipw})$. This equation corresponds to eq. 2 in the main manuscript assuming that D_{lipw} of a charged species is ten times smaller than that of the corresponding neutral species (eq 5). [j] Experimental (this study).

Metoprolol

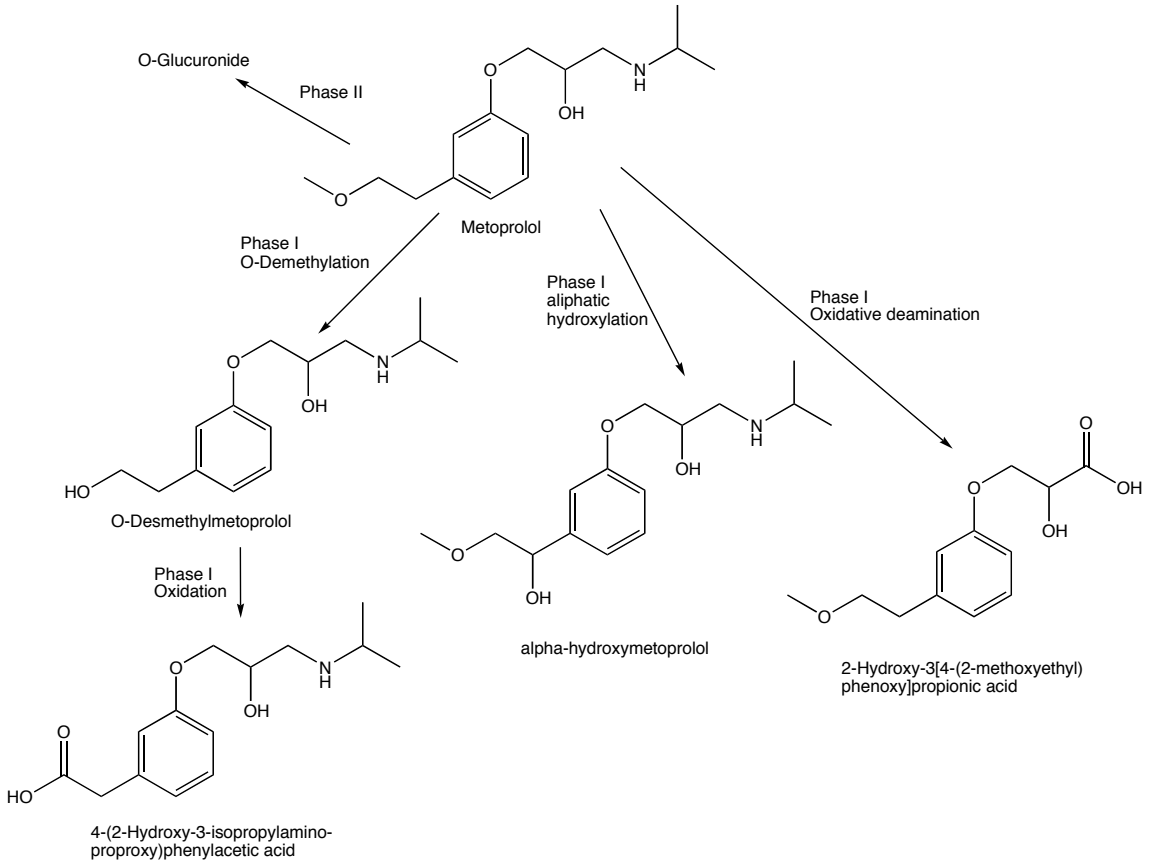

Figure S-6. Reaction scheme of human metabolism of metoprolol.

Table S-.5 Product distribution of metabolites in human excretory products and physicochemical descriptors of the metabolites of metoprolol.

Metabolite[a]	Fraction in feces (%min to %max)[a]	Fraction in urine (%min to %max)[a]	$\log K_{ow}$	$\log K_{lipw}$ (neutral species)[e]	Speciation at pH 7	$\log D_{lipw}$ (pH7)[f]
Metoprolol	0 to 5	3 to 10	1.88[b]		base, 99% positively charged	1.43[g]
Metoprolol-glucoronide		0	0.72[c]	1.17	neutral	1.17
O-Desmethyl-metoprolol		1	1.22[d]	1.62	equal to metoprolol	0.63
4-(2-Hydroxy-3-isopropylamino-proproxy)phenyl-acetic acid		65	1.09[d]	1.50	zwitterionic (aliphatic amine and carboxylic acid)	1.50
Alpha-hydroxymetoprolol		10	0.01[d]	0.52	equal to metoprolol	-0.47
2-Hydroxy-3[4-(2-methoxyethyl)phenoxy]propionic acid		10	1.14[d]	1.55	carboxylic acid	0.55

[a] Data from ref. (15,17,18) and Swiss Drug Kompendium (Documed AG, Basel, Switzerland, www.kompendium.ch, last visit to website: 07.11.2005). [b] Data from ref (26).[c] Estimated from K_{ow} of parent compound and K_{ow} for glucoronic acid (-2,57 (23)). [d] Estimated with the fragment method described in (23,24). [e] Calculated with eq. 4 of the main manuscript $\log K_{lipw} = 0.904 \cdot \log K_{ow} + 0.515$ (25). [f] Corrected for the speciation, assuming $\log D_{lipw}(pH7) = \log(f_{neutral} \cdot K_{lipw} + f_{charged} * 0.1 \cdot K_{lipw})$. This equation corresponds to eq. 2 in the manuscript assuming that D_{lipw} of a charged species is ten times smaller than that of the corresponding neutral species (eq 5). [g] Experimental (this study).

Atenolol

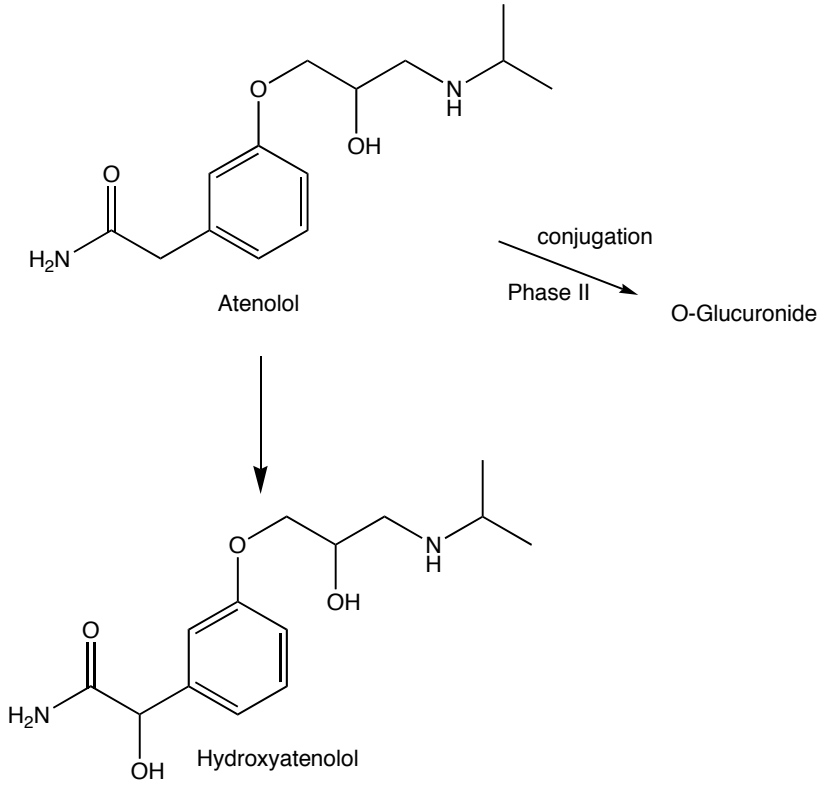

Figure S-7. Reaction scheme of human metabolism of atenolol.

Table S-6. Product distribution of metabolites in human excretory products and physicochemical descriptors of the metabolites of atenolol.

Metabolite[a]	Fraction in feces (%min to %max)[a]	Fraction in urine (%min to %max)[a]	$\log K_{ow}$	$\log K_{lipw}$ (neutral species)[e]	Speciation at pH 7	$\log D_{lipw}$ (pH7)[f]
Atenolol	36 to 56	33 to 40	0.16[b]		base, 99% positively charged	0.51[g]
Atenolol-glucoronide	0	0.8 to 4.4	-1.00[c]	-0.39	neutral	-0.39[h]
Hydroxyatenolol	0	1.1 to 4.4	-1.71[d]	-1.03	equal to atenolol	-2.02[h]

[a] Data from ref. (15,17,18,27). [b] Data from ref (26).[c] Estimated from K_{ow} of parent compound and K_{ow} for glucoronic acid (-2,57 (23)). [d] Estimated with the fragment method described in (23,24), using $f_{OH} = -1.64$ and $f_H = 0.23$. [e] Calculated with eq. 4 of the main manuscript $\log K_{lipw} = 0.904 \cdot \log K_{ow} + 0.515$ (25). [f] Corrected for the speciation, assuming $\log D_{lipw}(pH7) = \log(f_{neutral} \cdot K_{lipw} + f_{charged} * 0.1 \cdot K_{lipw})$. This equation

corresponds to eq. 2 in the manuscript assuming that D_{lipw} of a charged species is ten times smaller than that of the corresponding neutral species (eq 5). [g] Experimental (this study). [h] Note that for compounds of such low hydrophobicity, the QSARs are not valid any more. However, since the overall fraction of these metabolites is only 2 to 9%, an error in the D_{lipw} will not strongly alter the overall mixture effect.

Sotalol could not be assessed with this method because it did not show any effect in the chlorophyll fluorescence test therefore we have no reference data for the modeling.

Toxicity reduction by metabolism in the 30-min bioluminescence inhibition test with *V. fischeri*

In the 30-min bioluminescence inhibition test with *V. fischeri* all ß-blockers acted as baseline toxicants. The metabolite analysis performed analogously to the chlorophyll fluorescence assay resulted in a toxicity reduction that is depicted in Figure S-8. Metabolism had virtually no effect on atenolol and metoprolol, while the bacterial toxicity of propranolol was largely reduced by metabolism.

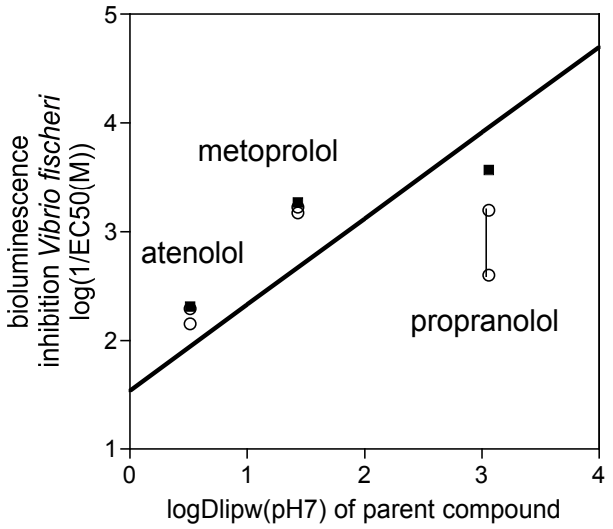

Figure S-8. Reduction of toxicity by metabolism. Effect concentrations EC50 in the bioluminescence inhibition test with V. fischeri *(expressed as negative logarithm of the EC50) as a function of lipophilicity of the parent compound expressed as $logD_{lipw}(pH7)$.* ■ *EC50 of the parent compounds;* ○ *realistic scenario (1): apparent EC50 of the metabolite mixture assuming baseline toxicity of the metabolites, $EC50_{apparent,\ baseline}$; the two points represent the range from $f_{excreted,imin}$ to $f_{excreted,imax}$.*

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