Materials and Methods

Culture of microbial cells: Axenic cultures of *P. putida* KT2440 UWC1 were prepared on nutrient agar and used to prepare an inoculum in 24 mL R2A medium (19h, 20°C, 200 rpm), with an $OD\lambda_{470nm}$ = 1.0 AU. This was used to inoculate experimental cultures at 5 µL mL^{-1}. R2A medium: (g L^{-1}): yeast extract (0.5), protease peptone (0.5), casamino acids (0.5), glucose (0.5), soluble starch (0.5), Na pyruvate (0.3), K_2HPO_4 (0.3), $MgSO_4 \cdot 7H_2O$ (0.05), pH 7.2 (KH_2PO_4). Pharmaceuticals were obtained from Sigma Aldrich, Gillingham, UK. Standard solutions of the pharmaceuticals were prepared at 1.25 mg mL^{-1} and filter sterilized.

Experiments were carried out in shaken culture at a 25 mL scale in replicate 100 mL flasks containing 24 mL R2A medium. 1 mL of 1.25 mg mL^{-1} filter sterilized standard solutions of the individual pharmaceuticals were added to replicate flasks, giving final concentrations of 50 µg mL^{-1}, and 1 mL filter sterilized water was added to replicate flasks as a control. Where samples of culture were removed prior to standard addition (e.g. for OD measurement or initial sampling) the volume of standard added was adjusted accordingly. Cultures were inoculated with 125 µL *P. putida* $OD\lambda_{470nm}$ = 1.0 AU and incubated at 20 °C, 200 rpm (150 rpm) for 15 h (the end of the exponential growth phase) in an orbital incubator.

In a preliminary experiment the estimation of minimum inhibitory concentration (MIC) of the pharmaceuticals for *P. putida* KT2440 UWC1 was carried out by a tube dilution method. For each pharmaceutical, a triplicate series of tubes containing the drug in 10 mL R2A medium at concentrations of 2.5, 2.25, 2.0, 1.75, 1.5, 1.25, 1.0, 0.75, 0.5, 0.25,

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0.2, 0.1, 0.05 and 0 mg mL^{-1} was prepared. Tubes were inoculated with 50 µL of a 19 h inoculum of KT2440 ($OD\lambda_{470nm}$=1.0 AU) and incubated at 20 °C. At 24 h and 48 h growth in the tubes was estimated visually and recorded. Three replicates were inoculated with 100 µL filter sterilized water as a negative control. The MIC for acetaminophen was found to be 2.5 mg mL^{-1}, for atenolol >2.5 mg mL^{-1}, for diclofenac >2.5 mg mL^{-1}, for ibuprofen >1 mg mL^{-1}, for mefenamic acid >1 mg mL^{-1}, and for propranolol 0.5 mg mL^{-1}. In this study we exposed *P. putida* KT2440 to the pharmaceuticals at 50 µg mL^{-1}; although higher than measured environmental concentrations in the UK, this is a concentration at least ten fold below the minimum inhibitory concentrations established for the pharmaceuticals, and at which we had seen a measurable effect for exposure to propranolol in earlier experiments using FT-IR spectroscopy. Having optimized the exposure concentrations for propranolol the same exposure concentrations were selected for all other drugs.

In order to determine the effect of each pharmaceutical on growth, the growth rates for *P. putida* exposed to each pharmaceutical at 50 µg mL^{-1} or water as a control were measured, initially using a Bioscreen C 200 microbiology workstation (Oy Growth Curves AB Ltd., Finland). Using a Bioscreen 100 well plate, 5 replicate wells containing 200 µL R2A medium containing each pharmaceutical at 50 µg mL^{-1} were inoculated with 2 µL of a 19 h inoculum of *P. putida* ($OD\lambda_{470nm}$ = 1.0 AU) and incubated at 20 °C, with shaking. OD readings were taken using a broad-band filter at 30 minute intervals for 16 h, growth curves plotted and the growth rate calculated for the exponential growth phase using Microsoft Excel. Growth rates were also measured in shaken culture comparable to the metabolomics experiments. For *P. putida* exposed

to each pharmaceutical or water as a control, growth was measured in triplicate cultures at a 25 mL scale. OD readings were measured at 470nm for a 1:10 dilution of samples taken at hourly intervals over a 21 h period, growth curves plotted and the growth rate calculated for the exponential growth phase using Microsoft Excel.

Monitoring recovery of the pharmaceuticals by HPLC: The recovery of the pharmaceuticals was monitored by HPLC in order to assess if there was any metabolism of the pharmaceuticals by *P. putida* over 24 h, and to observe the stability of the drugs in the culture medium. The culture of cells for the HPLC analysis was carried out in replicate at a 25 mL scale. For each pharmaceutical at a concentration of 50 μg mL^{-1} and water as a control, 6 replicate flasks were set up, and 3 of the replicate flasks were inoculated with *P. putida* and incubated at 20 °C, 200 rpm for 24 h. The remaining 3 replicates were inoculated with 125 μL filter sterilized water as negative controls. At 24 h the cultures (20mL) and controls were harvested and centrifuged (7697 *g*, 4 ºC, 3 min). 2 mL supernatant was filtered through a 0.2 μ syringe filter for HPLC. The cells were washed with water three times, and extracted with methanol (1.0 mL) with 3 cycles of freeze-thawing and sonication (samples were frozen in liquid nitrogen, thawed at 20 °C, sonicated for 3 min and vortexed thoroughly (L&R T9 sonicator, L&R Manufacturing, NJ, US). After centrifugation in a chilled centrifuge (7697 x g, 4 ºC, 3 min) 500 μL of the methanol supernatant was added to 2.0 mL methanol for HPLC, representing a 4 fold concentration of the original culture. HPLC was performed using an Agilent HPLC system (Agilent, Stockport, UK) on a Phenomenex Sphereclone ODS column (250 x 4.6 mm, 5 micron, Phenomenex, Macclesfield, UK). Water and acetonitrile were chromatography grade and supplied by Sigma Aldrich, Gillingham,

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UK. Pharmaceutical compounds, formic acid, potassium phosphate monobasic (KH_2PO_4) and orthophosphoric acid were obtained from Sigma Aldrich. Phosphate buffer (pH 3.8) was prepared by diluting KH_2PO_4 3.13 g in 1L water and adjusting the pH with orthophosphoric acid 10%. Acetaminophen, diclofenac, ibuprofen and mefenamic acid were analysed using water and acetonitrile containing 0.1% formic acid with a flow rate of 0.5 mL min^{-1} and a gradient of 10-100% acetonitrile over 0-10 min followed by 10 min at 100% acetonitrile. atenolol and propranolol were analysed with a gradient of acetonitrile and phosphate buffer pH 3.8.[1] The acetonitrile content of the mobile phase was increased linearly from 10 to 50% over 20 min followed by 7 min at 50% acetonitrile. Six point standard curves were calculated for the APIs at concentrations of 5, 10, 30, 50, 70 and 90 μg mL^{-1}. The standard curves with a linear trendline through the origin were calculated in Microsoft Excel on the basis of mean peak area. Where standards at the lowest concentrations were not in the range giving a linear response the line was recalculated for the four highest concentrations. Experimental values were calculated in Excel using y = mx + c.

The culture of cells for the FT-IR and GC-MS analysis was carried out in replicate at the 25 ml scale described above in 5 replicate flasks containing each drug at 50 μg mL^{-1}. Cultures inoculated with *P. putida* were incubated at 20 °C, 200 rpm for 15 h in an orbital incubator. Sample inoculation was randomized between classes and staggered to enable fast and reproducible sample processing. At the end of the exponential growth phase (15 h) the cells were harvested as described below for FT-IR and GC-MS as rapidly as possible; quenching was not performed in this experiment due to previously observed fragility of cells grown with the pharmaceuticals. (Indeed, initial

measurements for ATP utilizing a quenching and extraction method showed up to 90% of the ATP to be present in the quenching supernatant rather than the subsequent methanol cell extract, and cells were later harvested for ATP measurement using a direct quenching and extraction method in 100% Methanol at -48 °C.) During sample processing samples were kept on ice and centrifugation steps were carried out in chilled centrifuges in order to minimise further cell metabolism.

Metabolite fingerprinting of whole cells by FTIR spectroscopy was carried out according to a modified method of Goodacre *et al.*.[2] For FT-IR analysis 1.9 mL culture was removed to centrifuge tubes and centrifuged in a chilled centrifuge (7697 *g*, 4º C, 3 min, accuSpin Micro R; Fisher Scientific) and the supernatant discarded. The cells were washed with ice cold water (2 x 0.5 mL), centrifuged and the supernatant discarded. Cells were snap frozen in liquid nitrogen and stored at -80 °C prior to analysis. Frozen cells were thawed, resuspended in 25-50 µL water, the amount calculated to give an IR absorbance between 0.4 and 1.4 AU at 1665 cm^{-1}. 5µL samples were randomized and spotted on a silicon FT-IR plate. The samples were dried at 50°C for 30 min. Triplicate FT-IR spectra were collected on a Bruker Equinox 55 FT-IR spectrometer using OPUS software version 4. Spectra were collected in absorbance mode, from 4000-600 cm^{-1}, with a resolution of 4 cm^{-1}, with a sampling time of 64 scans. Spectra were baseline corrected using rubber-band baseline correction using the instrument software and exported in ASCII format for analysis in MATLAB.[3] Within MATLAB CO_2 absorbances were removed, the individual spectra were scaled between zero for the lowest recorded and 1 for the highest recorded absorbances. Data corresponding to

2826-1774cm^{-1} were also removed as there is no biologically relevant information in this part of the spectrum, and the variables scaled to unit variance.

Metabolite profiling of methanol cell extracts by GC-MS was carried out according to a modified method of Winder *et al.*[4] using GC-MS conditions optimized for yeast.[5] For GC-MS analysis 17 mL culture was transferred to a centrifuge tube, centrifuged in a chilled centrifuge (2521 *g*, 4 ºC, 10 min, CR322; Jouan) and the supernatant removed and discarded. The cells were washed in ice cold water (2 x 0.5 mL) and transferred to centrifuge tubes, centrifuged in a chilled centrifuge (7697 *g*, 4 ºC, 3 min) and the supernatant removed and discarded. Cells were snap frozen in liquid nitrogen and stored at -80 °C. The cells were subsequently extracted with 0.5 mL cold methanol (kept on dry ice (-78.5 °C)) through 3 freeze-thaw cycles as described above. After centrifugation (7697 x g, 4 ºC, 3min) the methanol was removed to clean 1.5 mL centrifuge tubes. The lysed cells were extracted with a further 0.5 mL cold methanol, centrifuged and the supernatant combined with the first extract. 100 µL succinic d_4 acid (0.083 mg mL^{-1}) was added as an internal standard. The samples were dried *in vacuo* in a HETO VR MAXI vacuum centrifuge attached to a HETO CT/DW 60E cooling trap (Thermo Life Sciences, Basingstoke UK), and stored at -80°C prior to derivatization for GC-MS. Standards of the individual pharmaceuticals containing 0.25 mg of each pharmaceutical and 100 µL internal standard solution, were prepared and dried. Standard mixtures of the pharmaceuticals, containing 1.0, 0.75, 0.5, 0.25, 0.1 and 0.01 mg of the pharmaceuticals, containing 100 µL internal standard solution, were also prepared and dried. The samples were derivatized for GC-MS with O-methylhydroxylamine hydrochloride (40 µL, 20 mg mL^{-1} in pyridine, 40°C, 80 min)

followed by N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) (40 µL, 40°C, 80min). 20 µL of a retention index solution (0.6 mg mL^{-1} n-C10, n-C12, n-C15, n-C19 and n-C22 alkanes in pyridine) was added prior to analysis. Samples were randomized and triplicate analytical samples were run on a GC-TOF-MS instrument (Agilent 6890N gas chromatograph and LECO Pegasus III TOF mass spectrometer) using the manufacturer's software (ChromaTOF version 2.15) and an optimized method developed for yeast. Data processing was performed as previously described[6], applying the ChromaTOF software, to construct a data matrix of metabolite peak vs. sample and infilled with peak areas for metabolites detected. The S/N threshold was set at 10, baseline offset at 1.0, data points for averaging at 3, and peak width at 3s. A database of metabolites present in typical sample classes was constructed from samples with each pharmaceutical and control. All peaks detected in samples that were present in the metabolite database were subsequently matched on retention index and mass-spectrum. A matrix of response ratios to the internal standard (peak area metabolite / peak area internal standard) was generated for peaks with a database match >750 and retention index match +/- 10. Since a metabolite may not be detected in all analytical replicates of a sample there is a need to fill in missing values in the matrix of response ratios. Where a metabolite was present in two out of the three analytical replicates, the mean of the two values detected was used to replace the missing value. Where a metabolite was detected in only one out of the three analytical replicates, this value was replaced with zero. Additionally, data for any metabolite was only retained if it was present in 75% of the biological replicates for any one class, resulting in a dataset for 150 metabolites. Furthermore, metabolites were excluded from the univariate statistical analyses for each pharmaceutical if absent from both the samples exposed to the drug and the samples

exposed to water. Variables (the peak ratios for each metabolite) were scaled to median absolute deviation because GC-MS data is especially skewed, i.e. not a normal distribution.

ATP Analysis: In an experiment conducted at a 25 mL scale, 12 replicate cultures were inoculated with 125 µL *P. putida* KT2440 ($OD\lambda_{470nm}$ = 1.0 AU) and incubated at 20 °C, 150 rpm. OD measurements at λ_{470nm} were taken throughout the experiment including the sampling times. At 10.5 h, approximately half-way through the exponential growth phase ($OD\lambda_{470nm}$ = 0.9AU), 0.9 mL culture sample was taken and quenched and extracted in 0.9 mL 100% methanol at -48 °C. Samples were snap frozen in liquid nitrogen and stored at -80 °C overnight. At 11 h, a 1.25 mg mL^{-1} filter sterilized standard solution of propranolol was added to 6 replicate flasks, to give a final concentration of 50 µg mL^{-1}, and filter sterilized water was added to 6 replicate flasks as a control. Both sample inoculation and propranolol and water addition were randomized between classes, and staggered to enable fast and reproducible sample processing. At 12 h (1 h after propranolol addition) 0.9 mL culture was harvested as described above. Samples were centrifuged (7697 *g*, 4 ºC, 3min) and the supernatant removed to a new centrifuge tube. Samples were standardized to unit OD, transferring an aliquot to new centrifuge tubes, the volume taken being inversely proportional to the $OD\lambda_{470nm}$ measurement taken at the time of sampling. Samples were dried in a vacuum centrifuge as described above and stored at -80°C for further analysis. ATP concentration was measured using a bioluminescence assay kit available from Roche Molecular Biochemicals (Roche Diagnostics, Burgess Hill UK) and following the manufacturer's instructions. For ATP measurement samples were reconstituted in 200 µL water, and 50

192 µL was diluted 2-fold, duplicate 40 µL aliquots were mixed with 40 µL luciferase
193 reagent and 10 s readings taken in a TD-20/20 luminometer (Steptech Instrument
194 Services, Stevenage, UK). A standard curve was obtained for ATP at concentrations
195 ranging from 10^{-6} to 10^{-11} M and experimental values calculated using Microsoft Excel.
196
197 *Statistical Analysis:*
198 *A combined principal components-canonical variates analysis* (PC-CVA) was carried
199 out for both the FT-IR spectra and GC-MS data using programs written in MATLAB as
200 detailed elsewhere [2]. PCA was carried out initially for 100 PCs as described in Jolliffe[7]
201 and CVA was carried out as described elsewhere .[8, 9] CVA is a supervised method that
202 allows groups in the data to be defined. CVA classes were defined according to the 15
203 replicates (5 replicate cultures x 3 instrument replicates, 12 for GC-MS for which a
204 small number of samples did not chromatograph well). The CVA was validated by
205 dividing the data into training and test sets and varying the number of PCs used in the
206 analysis. An n-fold interchange of training and test data (for FT-IR n=5; for GC-MS
207 n=4) was employed and the optimum number of PCs was selected where clustering of
208 the data was seen according to the classification, and where the PC-CV scores for the
209 test set when projected into PC-CVA fell within the bounds of the data for the training
210 set.
211
212 *ANOVA analysis:* Anova was carried out on the GC-MS data between cells exposed to
213 water and cells exposed to each pharmaceutical. ANOVA was carried out using
214 programs written in Matlab and described elsewhere.[10] The family-wise error rate
215 (FWER) was used to determine a threshold for significance for independent or

positively correlated variables.[11] The area under the receiver operator characteristic curve (ROC), considered to be one of the best means by which to describe the utility of a variable in binary classification was also calculated.[12] If the area under the ROC curve (AUC) is 0.5 then the variable is distributed similarly between case and control; for a metabolite entirely diagnostic of the class the AUC is 1. Results were visualised as a plot of the AUC *vs.* the p-value, and metabolites with an ROC > 0.85, with $p < 0.01$ selected for correlation analysis.

Correlation Analysis was carried out for significant metabolites identified from the ANOVA analysis of GC-MS data, observing correlations between metabolites in *P. putida* exposed to water, and contrasting these with correlations in *P. putida* exposed to individual pharmaceuticals. Even under uniform experimental conditions, the concentrations of metabolites in a metabolomic data set show a degree of variability between biological replicates. Metabolite concentrations do not vary independently, but are highly interconnected via metabolic correlation networks.[13] Firstly, inevitable small differences in enzyme concentrations, reflecting differences in gene expression, affect metabolite concentrations and result in interdependencies between metabolites. Secondly, cellular metabolism is influenced by a number of environmental factors, such as light intensity or nutrient supply. Rapidly changing diminutive differences, even in an approximately constant environment, result in changes in metabolite concentrations, which propagate through the network and result in a specific pattern of correlations. Correlation between two metabolites is the combined result of many biochemical reactions, regulatory interactions and the inducing fluctuations that regulate the system. The pair-wise correlation network represents a snapshot of the physiological state of the

sample at a given point in time. Systematic comparison of correlations across different experimental conditions can reveal invariant features of cellular metabolism. Likewise, changes in correlations can point to key points at which regulation has changed.[14] Correlation analysis was carried out for significant metabolites, with an area under the ROC curve > 0.85, the inputs for the analysis again being the median-scaled GC-MS peak responses. Correlation analysis was carried out using Graphviz open source graph visualization software[15] following an approach proposed by Kamada and Kawai[16], where an ideal spring is placed between every pair of nodes such that its length is set to the shortest path distance between the endpoints. The spring constant is proportional to the correlation between nodes and the geometric distance between nodes approximates their path distance in the graph. In statistics, this algorithm is also known as multidimensional scaling and its application to graph drawing was noted by Kruskal and Seery.[17] Here, the nodes are the identified significant metabolites and edges only exist between two nodes if the Spearman's rank correlation coefficient is >0.8. For the correlation analysis the critical p-value was set to the more usual value of 0.01. The reasoning behind this is that if several metabolite peaks are highly correlated (and possibly biologically linked) and also significant biomarkers (at the <0.01 level) then it is unlikely that they are false discoveries.

ANOVA analysis was carried out for the ATP concentration prior to and 1 hour after exposure in cells exposed to water as a control and in cells exposed to propranolol. The critical p-value for rejecting the null hypothesis in a single test selected was the more usual value of 0.01.

Results and Discussion for the determination of minimum inhibitory concentrations, the effect of each pharmaceutical on growth and monitoring recovery of the pharmaceuticals by HPLC.

Initially the growth curves for *P. putida* exposed to the pharmaceuticals were measured using a Bioscreen C200 workstation. The mean growth rate for the control cultures was 0.269 ± 0.002 h^{-1}, for *P. putida* exposed to acetaminophen 0.264 ± 0.002 h^{-1}, atenolol 0.265 ± 0.003 h^{-1}, diclofenac 0.267 ± .004 h^{-1}, ibuprofen 0.267 ± .003 h^{-1}, mefenamic acid 0.266 ± .005 h^{-1} and propranolol 0.262 ± 0.001 h^{-1}. The mean OD at the end of the exponential growth phase (8h) for the control cultures was 0.493 ± 0.008 AU, for cultures exposed to acetaminophen 0.488 ± 0.009 AU, atenolol 0.492 ± 0.105 AU , diclofenac 0.491 ± 0.005 AU, ibuprofen 0.493 ± 0.006, mefenamic acid 0.492 ± 0.004 AU and propranolol 0.480 ± 0.006 AU.

In shaken culture comparable to the metabolomics experiments, the mean growth rate for the control cultures was 0.517 ± 0.027h^{-1}, for *P. putida* exposed to acetaminophen 0.516 ± 0.011 h^{-1}, atenolol 0.502 ± 0.015 h^{-1}, diclofenac 0.517 ± 0.012 h^{-1} ibuprofen 0.510 ± 0.022 h^{-1}, mefenamic acid 0.503 ± 0.008 h^{-1} and propranolol 0.494 ± 0.015 h^{-1}. The mean OD (1:10 dilution) at the end of the exponential growth phase (15h) for the control cultures was 0.170 ± 0.004 AU, for cultures exposed to acetaminophen 0.155 ± 0.004 AU, atenolol 0.156 ± 0.003 AU, diclofenac 0.172 ± 0.003 AU, ibuprofen 0.162 ± 0.002, mefenamic acid 0.170 ± 0.002 AU and propranolol 0.142 ± 0.003 AU.

Recovery of the pharmaceuticals from cultures of *P. putida* was monitored by HPLC with quantification based on HPLC peak area and comparison with external standards used in producing a standard curve. Propranolol was the only pharmaceutical detected in

the methanol cell extracts; a total of 38.4 μg mL^{-1} was detected in the *P. putida* culture supernatant and cell extract combined compared to 43.4 μg mL^{-1} in the sterile control. This small change in concentration (< 10%) indicates little metabolism of propranolol by *P. putida* or degradation in solution over 24h. The remaining pharmaceuticals were recovered completely from the culture supernatants compared with the sterile controls at concentrations > 41μg mL^{-1}, indicating little microbial degradation, or chemical degradation in solution.

Multivariate Analysis of GC-MS Data: Cross-validated PC-CVA models were generated for the GC-MS data of *P. putida* exposed to pharmaceuticals at 50 μg mL^{-1}. PC-CVA was performed for 6 pharmaceuticals and control using 4 PCs selected after a 4-fold interchange of training and validation sets, as detailed above. Figure SI1a shows the PC-CV score 1 plotted against PC-CV score 2 and shows extracts from *P. putida* exposed to propranolol separated from remaining classes along PC-CV1, for cells exposed to acetaminophen along PC-CV1 and PC-CV2, and for cells exposed to mefenamic acid along PC-CV2. The model showed greater discrimination for *P. putida* exposed to propranolol and acetaminophen than any between the remaining pharmaceutical classes and control. Models were generated which discriminate between individual classes and control (shown for ibuprofen in SI3 Figure 1b).

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