The chemotherapeutic drug methotrexate selects for antibiotic resistance

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20 **Abstract**

21 **Background:** Understanding drivers of antibiotic resistance evolution is fundamental for
22 designing optimal treatment strategies and interventions to reduce the spread of antibiotic
23 resistance. Various cytotoxic drugs used in cancer chemotherapy have antibacterial properties,
24 but how bacterial populations are affected by these selective pressures is unknown. Here we
25 test the hypothesis that the widely used cytotoxic drug methotrexate affects the evolution and
26 selection of antibiotic resistance.

27 **Methods:** First, we determined methotrexate susceptibility (IC_{90}) in a collection of clinical
28 strains with and without pre-existing trimethoprim resistance determinants and the co-selective
29 abilities of methotrexate by growth curve measurements. We constructed fluorescently labelled
30 isogenic pairs of *E. coli* MG1655 differing only in trimethoprim resistance determinants
31 (chromosomal *folA* mutations and plasmid-mediated *dfrA* genes) and determined the minimum
32 selective concentrations of methotrexate using flow-cytometry. We further used an
33 experimental evolution approach to investigate the effects of methotrexate on *de novo*
34 trimethoprim resistance evolution in a clinical *E. coli* strain.

35 **Findings:** We show that methotrexate can select for acquired trimethoprim resistance
36 determinants located on the chromosome or a plasmid in clinical strains of *E. coli*. Additionally,
37 methotrexate co-selects for genetically linked resistance determinants when present together
38 with trimethoprim resistance on a multi-drug resistance clinical plasmid. These selective effects
39 occur at concentrations 40- to >320-fold below the methotrexate minimal inhibitory
40 concentration for *E. coli* and is independent of the well characterized efflux mediated intrinsic
41 resistance mechanisms.

42 **Interpretation:** Our results strongly suggest a selective role of methotrexate for virtually any
43 antibiotic resistance determinant when present together with trimethoprim resistance on a

multi-drug resistance plasmid. The presented results may have significant implications for patient groups strongly depending on effective antibiotic treatment.

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Research in context

Evidence before this study: We searched PubMed and Web of Science for the following search terms: Methotrexate + trimethoprim + resistan*; Cytostatic* + resistan* + antibiotic*; Cytostatic* + cross + resistan* + antibiotic; Chemotherapy + driving + resistan* + antibiotic*; drivers/driving + antimicro* + resistan* in May 2020. The evidence indicate that cytostatic drugs, including methotrexate, a widely used drug for treatment of cancer and inflammatory diseases, share molecular targets with commonly used antibiotics (e.g. trimethoprim). Further, it is proposed that cytostatics may drive antibiotic resistance evolution due to microbiome alterations, overlapping intrinsic resistome, and SOS induced mutagenesis. Important questions remain regarding direct evidence for cross-resistance development between cytostatics and antibiotics, and how these affect the selection, and spread of acquired antibiotic resistance determinants.

Added value: To our knowledge, we show for the first time that methotrexate directly selects for acquired trimethoprim resistance determinants on the chromosome as well as on a clinical multi-drug resistance plasmid. These selective and co-selective effects occur at low methotrexate concentration ranges expected to represent intestinal concentrations during clinical use.

Implications: The presented data show that methotrexate has the potential to select for virtually any given antibiotic resistance gene when genetically linked to trimethoprim resistance**.** This study highlights the need for increased awareness of the presence of acquired antibiotic

69 resistance determinants in the gut of patients with impaired immunity undergoing methotrexate
70 treatment to preserve the effects of downstream antibiotic treatments.
71
72 **Introduction**
73 Antibiotic resistance is a major risk factor for patients with impaired immunity, such as cancer
74 patients, and often a patient's survival depends on antibiotic treatment to reduce the risk for
75 hospital-acquired infections during chemotherapy[1]. Several cytotoxic drugs used in cancer
76 chemotherapy are known to both elevate bacterial mutation rates and have direct antimicrobial
77 properties[2]. It has been proposed that cancer chemotherapy may drive *de novo* antibiotic
78 resistance evolution through SOS induced mutagenesis[3], and some reports have provided
79 support for this hypothesis[4,5]. Recently, the effects of non-antibacterial drugs on bacteria
80 typically found in the human gut were thoroughly explored and cytotoxic drugs were reported
81 to cause the most severe alterations of the microbiota[6]. Taken together, these studies suggest
82 that cytotoxic drugs affect survival of human gut commensals, they may increase the
83 evolvability of bacterial populations, and lead to reduced susceptibility towards drugs used to
84 treat cancer in bacteria. How bacterial populations respond to selective and co-selective
85 pressures exerted by individual cytotoxic drugs and the implications for antibiotic resistance
86 selection and spread is unknown. Thus, there is an urgent need to understand these potential
87 collateral effects of cancer chemotherapy to ensure effective antibiotic treatment for a large
88 group of immunocompromised patients. Moreover, cytotoxic drugs may constitute a previously
89 unrecognized target for intervention to limit the selection and spread of antibiotic resistance.
90
91 Methotrexate (MTX) is widely used in treatments including but not limited to; cancer of the
92 breast, skin, head, neck, and lung as well as many inflammatory diseases, such as rheumatoid
93 arthritis[7]. We specifically targeted resistance towards trimethoprim (TMP), since both drugs act

through inhibiting the dihydrofolate reductase enzyme in bacteria and eukaryotic cells, central in DNA synthesis[8]. TMP in combination with sulfamethoxazole is among the most frequently used antibiotics in the treatment of urinary tract infections and is recommended as first line treatment internationally[9]. Our target organism in this study is *Escherichia coli*, the most common agent of nosocomial infections world-wide[10]. *E. coli* is known to display intrinsic resistance towards methotrexate through AcrAB-TolC mediated efflux[11], however TMP is not a substrate for this efflux system.

Previous studies have focused on the abilities of MTX and other non-antibiotics to inhibit bacterial growth[6]. These approaches have provided valuable insights on the effects of non-antibiotics as modulators of the intestinal flora but lacked the necessary resolution to detect more subtle selective effects on acquired antibiotic resistance determinants.

Here, we hypothesize that despite the demonstrated *E. coli* intrinsic MTX resistance[11], MTX can affect antibiotic resistance evolution in *E. coli,* due to the shared molecular target with TMP. We show that MTX selects for acquired bacterial TMP resistance (TMP^R) and co-selects for other antibiotic resistance determinants when co-residing on a mobile genetic element. Exposure to a wide concentration range of MTX selects for mutations identical to those emerging during TMP selection in clinical isolates of *E. coli*. Moreover, we show that the minimum selective concentrations (MSCs) of MTX and positive selection for chromosomal and plasmid-mediated TMP^R determinants occurs at concentrations 40- and >320-fold below the MTX minimum inhibitory concentrations (MICs), respectively.

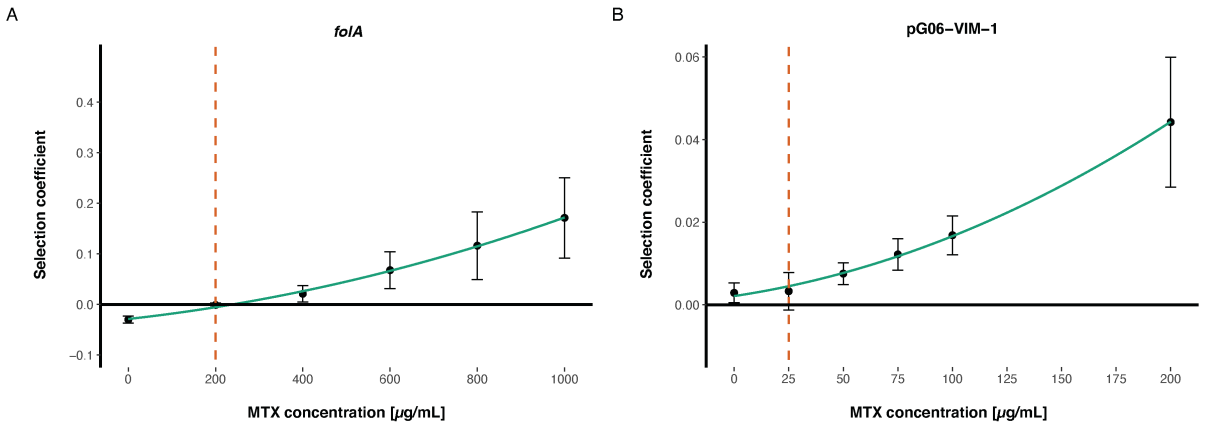
Methods

Bacterial strains and growth conditions: Bacterial strains used in this study and their assigned reference numbers are listed in Table S1. Most strain constructs were derived from the *E. coli* K-12 strain MG1655 (DA4201/DA5438), or for cloning purposes from *E. coli* DH5α

119 (MP18-09). Two isogenic pairs of clinical *E. coli* strains, where one strain is trimethoprim
120 susceptible (TMP^S) and the other is TMP^R, were used for fitness measurements. In the first pair,
121 TMP^R in *E. coli* K56-2 (MP06-01) is caused by one intragenic point mutation T>A (W30R) in
122 the *folA* gene, and one in its promotor region (P_{folA}, C>T 58 base pairs (bp) upstream of the
123 gene)[12,13] (MP06-05). In the second pair, *E. coli* K56-75 (MP06-41) harbouring the pG06-VIM-
124 1 multi-drug resistance plasmid represents the TMP^R counterpart (MP05-31). This plasmid
125 encodes, amongst seven other antibiotic resistance genes, two different *dfrA* genes (*dfrA1* and
126 *dfrA12*) as putative TMP^R determinants as well as a carbapenem resistance determinant (bla_{VIM-1})[14]. For accurate MSC measurements we constructed pairs of fluorescently tagged (*yfp* and
128 *bfp*) *E. coli* MG1655 strains containing the same resistance markers as described above. For
129 details concerning growth conditions, susceptibility testing and strain constructions see
130 protocols in supplementary information (SI).
131
132 **Fitness measurements:** Growth rates were determined using a BioscreenC MBR reader (Oy
133 Growth Curves Ab, Ltd). The growth rate calculations were done using the Bioscreen Analysis
134 Tool BAT 2.1[15] (see SI for detailed protocol). Competition experiments were performed in six
135 biological replicates, including dye swaps, using the fluorescently tagged strain pairs described
136 above, containing *folA* or pG06-VIM-1 mediated TMP^R. Both competition experiments, MSC
137 calculations and competitions performed at different starting ratios were done as previously
138 described in[16] (see SI for detailed protocols).
139
140 **Experimental evolution:** To examine the effect of MTX presence on TMP^R evolution, strain
141 K56-2 (MP06-01) was serially passaged in liquid cultures with MTX supplemented at a
142 concentration slightly above the estimated MSC. Initially, ten independent overnight cultures
143 were started from independent colonies on separate agar plates. From the original overnight

144 cultures, ~10^3 cells were used to start ten independent lineages in 1 mL batch cultures
145 containing 400 μg/mL MTX (lineages 1-10). Every 12 h for 25 days, the lineages were serially
146 passaged by 1,000-fold dilution, allowing for ~500 generations of growth. Every ~50
147 generations the populations were frozen down at –80°C for downstream analysis. For detailed
148 protocols concerning experimental evolution, selective plating on high concentrations of MTX
149 and whole genome sequencing see SI.
150
151 **Results**
152 **Methotrexate selects for pre-existing trimethoprim resistance determinants**
153 We determined the MICs of MTX in clinical and laboratory strains of *E. coli* (Table S1, SI).
154 Initial experiments revealed variable, but high MTX MICs (Table S2) consistent with previous
155 reports demonstrating that *E. coli* displays intrinsic resistance towards MTX due to AcrAB-
156 TolC mediated efflux[6,11]. We also observed that the MTX MIC was dependent on the presence
157 of TMP^R determinants. All isolates with a functional TMP resistance determinant and increased
158 TMP MIC showed consistently higher MTX MICs (>32 mg/mL) than TMP susceptible isolates
159 (4-32 mg/mL), indicating possible co-selective abilities of the two drugs.
160 Antibiotic resistance selection and co-selection have traditionally been assumed to occur
161 between the MICs of susceptible and resistant isolates within a bacterial population (known as
162 the selective window)[16]. However, several reports unequivocally show that antibiotic resistance
163 selection and co-selection can occur at concentrations several hundredfold below the MIC of a
164 susceptible isolate (known as sub-MIC)[16,17]. To test how sub-MICs of MTX affect bacterial
165 fitness, we measured exponential growth rates for two pairs of clinical isogenic TMP^R and
166 TMP^S *E. coli* across a wide MTX concentration span. One pair with TMP^R located on the
167 chromosome (two mutations associated with *folA*)[12] and one pair with TMP^R (*dfrA*) located on
168 the MDR plasmid pG06-VIM-1[14]. TMP^S strains displayed sharply declining growth rates

169 between 1 and 2 mg/mL of MTX, whereas the TMP^R strains remained unaffected (Figure S1).
170 These results suggest a selective benefit during MTX exposure for TMP^R strains at
171 concentrations below the observed MTX MIC of the TMP^S clinical isolates.
172 The MSC describes pharmacodynamically the lowest concentration where selection for
173 resistance occurs[16]. To determine the MSC for MTX, we constructed a fluorescently tagged
174 pair of *E. coli* MG1655 strains to enable accurate separation between the two in mixed
175 populations. In these backgrounds, we introduced TMP^R, either through mutations (*folA*) using
176 genome engineering or the pG06-VIM-1 plasmid. The isogenic TMP^R and TMP^S strain pairs
177 were competed head-to-head by serial passage for 30 generations and the ratio of TMP^R:TMP^S
178 was determined over time using flow cytometry. From this data the MSC was estimated (Tables
179 S3-S5)[16]. *E. coli* with chromosomal *folA* mutations had an MSC of 200 µg/mL (1/40 of the
180 MIC of MTX) whereas those harboring the plasmid were close to the detection limit of the
181 assay[16] and we conservatively estimated the MSC to be <25 µg/mL (less than 1/320 of the MIC
182 of MTX) (Figure 1). Taken together, our data strongly suggest that selection for TMP^R occurs
183 at MTX concentrations far below the estimated MTX MIC.
184



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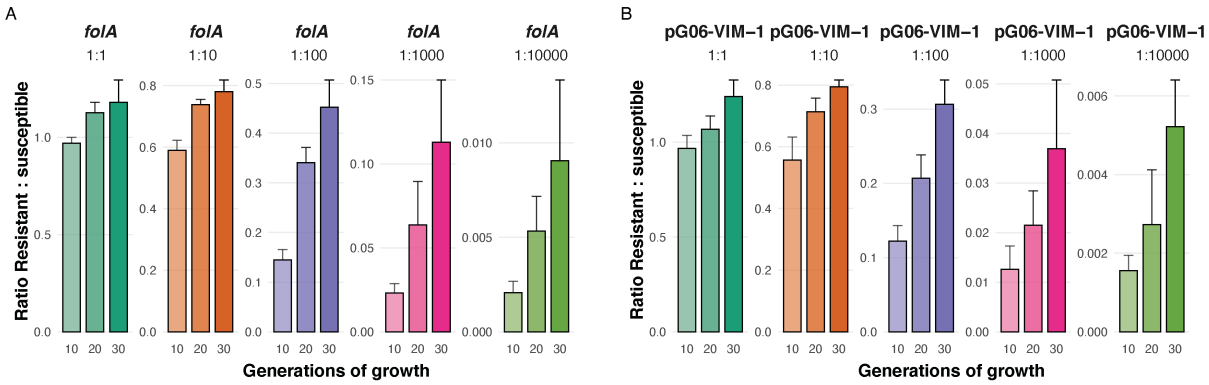
186 **Figure 1: Selection coefficients as functions of MTX concentrations from competition**
187 **experiments between TMP^R and TMP^S isogenic strains.** The MSC is defined as the
188 concentration where the selection coefficient equals zero. The MSC of *E. coli* MG1655

189 harboring **(A)** two chromosomal *folA* mutations (MP18-04 and MP18-07) is set to 200 µg/mL,
190 and **(B)** the MDR pG06-VIM-1 plasmid encoding *dfrA12* (MP18-05 and MP18-08) is
191 conservatively set at 25 µg/mL. Dashed lines represent the set MSC, bullets the average
192 selection coefficients and error bars the standard deviations.
193
194 **Sub-MICs of methotrexate promotes invasion of trimethoprim resistance determinants**
195 **even when rare in *E. coli* populations**
196 Exploring MTX-selective dynamics further, we asked if TMP^R determinants could invade the
197 population at lower initial densities to exclude potential bias from the 1:1 ratio in the
198 competition experiments. We started competition experiments from frequencies as low as 10^{-4}
199 of the TMP^R strains, at concentrations slightly above the estimated MSC of MTX (400 µg/mL
200 for *folA* mediated resistance and 75 µg/mL for pG06-VIM-1 mediated resistance) and followed
201 the change in ratios over 30 generations of growth (Figure 2). Both chromosomal and plasmid
202 mediated TMP^R determinants were able to invade, even when initially rare in their respective
203 populations, strongly suggesting that the MTX selective effects are independent on initial
204 frequencies of resistant and susceptible strains during competition experiments.
205



206
207 **Figure 2: Competition experiments during sub-MIC MXT exposure at different initial**
208 **frequencies of TMP^R strains.** The change in TMP^R:TMP^S ratios over 30 generations of growth

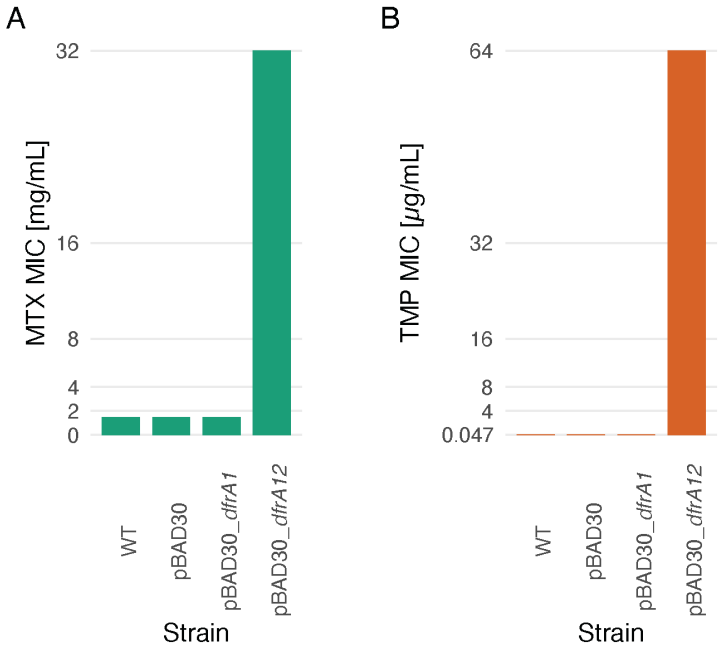
209 at MTX concentrations slightly above the MSCs where *E. coli* MG1655 harbors **(A)** two *folA*
210 mutations (MP18-07), and **(B)** the pG06-VIM-1 plasmid (MP18-08) were competed against a
211 differently tagged, isogenic susceptible strain (DA56507) at 1:1, $1:10^{-2}$, $1:10^{-3}$ and $1:10^{-4}$
212 starting ratios. Error bars represent the standard deviation of the average ratio.
213

214 **Methotrexate co-selects for resistance determinants on a multi-drug resistance plasmid**

215 The *dfr*-genes represent a common TMP^R mechanism in *E. coli* and these genes are frequently
216 located on mobile genetic elements such as integrons and plasmids. Given that MTX selects for
217 *dfr*-mediated TMP^R, co-selection of other genetically linked resistance genes is likely. To show
218 this, we used the MDR pG06-VIM-1 plasmid harboring *dfrA1* and *dfrA12* along with multiple
219 resistance determinants including four aminoglycoside resistance genes and the bla_{VIM-1}
220 carbapenemase gene conferring resistance to broad-spectrum β-lactams including
221 carbapenems[14]. To assess the stability of pG06-VIM-1 in our strains competing in the presence
222 of MTX, *E. coli* K56-75 harboring the plasmid (MP05-31) was serially passaged in batch
223 cultures with 400 µg/mL MTX supplemented for 50 generations. The lineages were then plated
224 on non-selective agar and 100 colonies from each lineage tested for reduced susceptibility
225 towards ampicillin, TMP, streptomycin and spectinomycin. The results revealed complete
226 phenotypic stability across all three lineages, confirming MTX mediated co-selection of
227 plasmid mediated MDR.

228 To verify that the TMP^R determinants on the MDR pG06-VIM-1 plasmid is the primary
229 mediators of MTX resistance and selection (Table S2), both *dfrA1* and *dfrA12* were isolated
230 from the plasmid and cloned onto an expression vector and the effects of the individual genes
231 measured. Of the two genes, only *dfrA12* was shown to give the same resistance pattern for
232 TMP as well as MTX as the pG06-VIM-1 plasmid (Figure 3), and the lack of detectable
233 phenotype for *dfrA1* (MP18-11) is likely due to a start codon frameshift mutation[14].

234



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236 **Figure 3: MTX and TMP MIC of *E. coli* DH5α expressing *dfrA1* or *dfrA12*.** The MTX **(A)**
237 and TMP **(B)** MIC for the wild type (WT) *E. coli* DH5α (MP18-09) compared to the strain
238 harboring the empty pBAD30 (MP18-10) expression vector as well as strains with pBAD30
239 with different *dfrA* genes expressed under the inducible expression control by the pBAD
240 promotor (MP18-11 and MP18-12). The detection limit of the assay is 64 µg/mL for TMP and
241 32 mg/mL for MTX. For both drugs, the MIC of *E. coli* DH5α expressing *dfrA*12 (MP18-12)
242 exceeded the detection limit whereas the strain expressing *dfrA1* (MP18-11) has the same MICs
243 as the WT strain. Showing that MTX and TMP resistance conferred by the pG06-VIM-1
244 plasmid is caused by the *dfrA12* gene.

245

246 **Methotrexate selects for *de novo* trimethoprim resistance**

247 We further examined whether exposure to MTX could lead to *de novo* TMP^R evolution. We
248 selected spontaneous mutants from overnight cultures with and without exposure to MTX,
249 plated on selective agar at high MTX concentrations and tested for TMP cross-resistance
250 (Figure S2, Table S6). *E. coli* K56-2 isolated at 16 and 32 mg/mL MTX (MP18-17 to MP18-

251 28) displayed increased MICs of TMP close to or above the clinical breakpoint[18], clearly
252 demonstrating selection for TMP^R by MTX. DNA sequencing of the resistant isolates revealed
253 two different mutations in the *folA* promoter, previously reported to result in TMP^R[19], as well
254 as a single mutation in the *marR* gene (Table S6-S11).
255 Finally, we asked if exposure to sub-MICs of MTX close to the estimated MSCs would select
256 for *de novo* TMP^R mutations in a susceptible *E. coli* population. Starting from 1,000 cells to
257 minimize the probability of pre-existing mutants, we grew ten independent lineages of the *E.*
258 *coli* K56-2 strain at 400 μg/mL MTX for 500 generations. The frequency of TMP^R was
259 determined every 50 generations. TMP^R ascended in frequency in 2/10 lineages at different
260 rates and time-points during the first 250 generations before they were outcompeted by a
261 different set of mutants with reduced susceptibility to MTX and no cross-resistance to TMP
262 (Figure 4, Table S12). These experiments show that MTX exposure can select for *de novo*
263 TMP^R, both at high and sub-MIC concentrations.
264

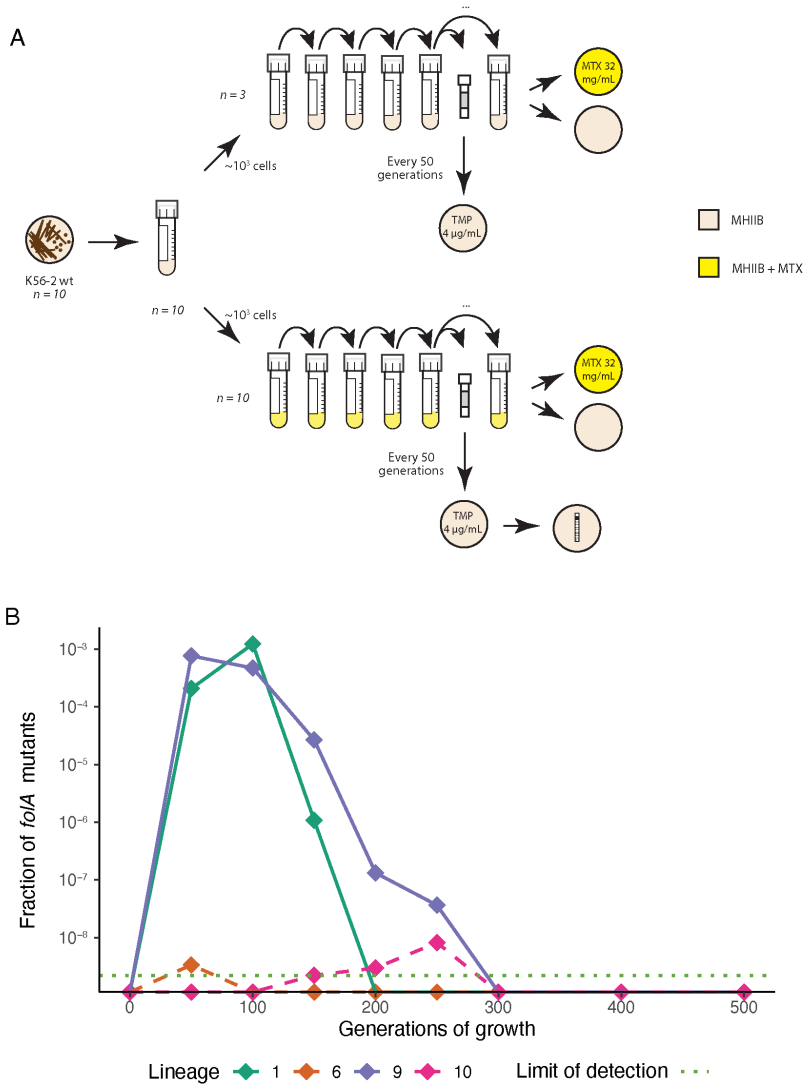

Figure 4: Evolution of TMP^R during MTX exposure for 500 generations. (A) Sub-MIC evolution experimental set-up. Ten biological replicates of K56-2 (MP06-01) were evolved for ~500 generations with 400 µg/mL MTX and three biological replicates without drug. All lineages were screened for TMP^R every 50 generations. After 500 generations all end-point populations were plated on 32 mg/mL MTX. All populations were able to grow at 32 mg/mL MTX, but not a single clone isolated conferred TMP^R, strongly suggesting that reduced susceptibility to MTX with no cross-resistance to TMP evolved in the endpoint populations. **(B)** Fractions of TMP^R *folA* mutants isolated every 50 generations from the lineages where these were detected. The detection limit of the assay was ~2x10^{-9}. Solid lines represent the two

275 lineages where TMP^R emerged and ascended in frequency whereas dotted lines indicate
276 spontaneous mutants.
277
278 **Pharmacokinetic approximations**
279 To assess pharmacokinetic relevance, we attempted to estimate the MTX concentration range
280 likely to be found in the intestine of patients undergoing MTX treatment. Limited information
281 is available on gut MTX concentrations following intravenous administration during cancer
282 treatment, as pointed out by others[6]. Pharmacokinetic data reveal that up to 90% of administered
283 MTX is renally excreted[20] and we assume that the remaining ~10% of the dose constitutes the
284 upper limit of the concentration range present in the human intestine. The lower limit is set to
285 2% of the dose based on the mean ^3H labelled MTX concentrations measured in stool samples
286 from nine patients receiving MTX intravenously[21]. From this, we set a 24 hour transition time
287 in a total volume of 0.6 L[6] and calculated the dose (*d*) required to achieve MSC in the human
288 intestine from:

289 $$\frac{d}{0.6L}\ x\ (0.1\ or\ 0{,}02) = MSC$$

290 Estimated doses needed to reach intestinal MSCs assuming 2% and 10% fecal MTX
291 concentrations were from 0.15g to 0.75g for plasmid-mediated TMP^R and from 1.2g to 6g for
292 chromosomal *folA* mutations. Thus, assuming close to $2m^2$ body surface in grown-up patients[22]
293 estimates of MSC for plasmid-mediated TMP^R translates to dosing regimens from 75-375
294 mg/m^2 and from 0.6-3 g/m^2 for the chromosomal *folA* mutations. These approximations indicate
295 that our MSC estimates are relevant for patients receiving high dose MTX treatment (1-12
296 g/m^2)[23]. A recent study used a different approach estimating gut MTX concentrations following
297 oral administration during treatment of rheumatoid arthritis[24]. Their data suggested MTX
298 concentrations as high as 100 μg/mL are found in the gut, suggesting that our estimated MSC

299 for plasmid-mediated antibiotic resistance determinants (25 μg/mL) is well within this
300 concentration range.
301
302 **Discussion**
303 Here we show that exposure to the cytotoxic drug MTX affects selection and evolution of TMP^R
304 determinants at clinically relevant concentrations. Notably, MTX can mediate selection of any
305 antibiotic resistance determinant when TMP^R is co-localized on a mobile genetic element across
306 a wide concentration gradient. Arguably, this potentially important side-effect of MTX
307 treatment has been previously unrecognized, as studies on the effects of non-antibiotic drugs,
308 including MTX, have either focused on bacterial growth inhibition or used drug concentrations
309 around the MIC[6,25-27], with a few exceptions[17,28].
310 Using the approaches outlined here, including high resolution mixed culture competition
311 experiments, allow determination of the true MTX selective window ranging from the MSC to
312 the MIC[16]. This is particularly relevant for non-antibiotics for which bacteria display reduced
313 susceptibility. In *E. coli,* MTX is a substrate for the AcrAB-TolC efflux pump[11] and selective
314 effects as those demonstrated here would not have been detected in classical susceptibility
315 and/or growth assays in bacterial monocultures. This was recently supported in an *E. coli*
316 chemical genetic screen where clear growth inhibitory effects of MTX, as well as for a range
317 of other non-antibiotics, were only demonstrated in a *tolC* knock-out mutant (i.e. in a mutant
318 lacking the intrinsic mechanism of resistance)[6].
319 Given that many cytotoxic drugs are structurally similar to antibiotics (e.g.
320 doxorubicin/tetracyclines), or target similar key processes as the major antibiotic groups (e.g.
321 DNA/protein synthesis) it is possible that cancer chemotherapy may lead to increased levels of
322 antibiotic resistance in a vulnerable patient group that very often rely on efficient antibiotic
323 treatment for survival. To acquire a deeper understanding of the evolutionary potential of novel,

324 non-antibiotic drivers of antibiotic resistance the approaches presented here are essential. These
325 approaches need to be combined with an improved understanding of the intestinal
326 pharmacokinetics of MTX and other cytotoxic drugs, possible effects of co-administered drugs
327 such as leucovorin mediated MTX rescue[29], and their interactions with the human microbiome.
328 Such knowledge could allow identification of antibiotic + non-antibiotic drug combinations
329 that should be avoided to preempt resistance evolution.
330 Recent studies have showed that non-antibiotics can increase mutation rates[4,5] and promote
331 horizontal gene transfer[28,30]. Taken together with the data presented here it is clear that non-
332 antibiotic drugs have the potential to affect the evolution, selection, and spread of antibiotic
333 resistance determinants. It is also clear that without a better understanding of these potential
334 drivers of antibiotic resistance, interventions including those targeting human consumption of
335 antibiotics may be negatively affected.
336
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344
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346 constructions were done by PT and JSG. Experiments were conducted by JSG, EGAF and
347 CIMK. WGS analysis was done using a bioinformatic pipeline designed by JH. WGS data was
348 verified by JH and JSG. Experimental data was verified by JSG and PJJ. Formal data analysis

349 and data visualization was done by JSG. The first draft of the manuscript was written by JSG
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352
353 **Competing Interests:** The authors declare no competing interests. **Data availability:** WGS
354 data are available at NCBI (BioProject PRJNA677979).
355
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