The chemotherapeutic drug methotrexate selects for antibiotic resistance

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Abstract

Understanding drivers of antibiotic resistance evolution is fundamental for designing optimal treatment strategies and interventions to reduce the spread of antibiotic resistance. Various cytotoxic drugs used in cancer chemotherapy have antibacterial properties, but how bacterial populations are affected by these selective pressures is unknown. Here we test the hypothesis that the widely used cytotoxic drug methotrexate affects the evolution and selection of antibiotic resistance through the same mechanisms as the antibiotic trimethoprim. We show that methotrexate can select for trimethoprim resistance determinants located on the chromosome or a plasmid in clinical strains of *Escherichia coli*. Additionally, methotrexate can co-select for virtually any antibiotic resistance determinant when present together with trimethoprim resistance on a multidrug-resistance clinical plasmid. These selective effects occur at concentrations 40- to >320-fold below the methotrexate minimal inhibitory concentration for *E. coli,* suggesting a selective role of methotrexate chemotherapy for antibiotic resistance in patients that strongly depend on effective antibiotic treatment.

Significance statement

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 resistance determinants in the gut of patients with impaired immunity undergoing methotrexate treatment to preserve the effects of downstream antibiotic treatments.

Introduction

Antibiotic resistance is a major risk factor for patients with impaired immunity, such as cancer patients, and often a patient's survival depends on antibiotic treatment to reduce the risk for hospital-acquired infections during chemotherapy[1,2]. Several cytotoxic drugs used in cancer chemotherapy are known to both elevate bacterial mutation rates and have direct antimicrobial properties[3,4]. It has been proposed that cancer chemotherapy may drive *de novo* antibiotic resistance evolution through SOS induced mutagenesis[5], and some reports have provided support for this hypothesis[6,7]. Recently, the effects of non-antibacterial drugs on bacteria typically found in the human gut were thoroughly explored and cytotoxic drugs were reported to cause the most severe alterations of the microbiota[8]. Taken together, these studies suggest that cytotoxic drugs affect survival of human gut commensals, they may increase the evolvability of bacterial populations, and lead to reduced susceptibility towards drugs used to

58 treat cancer in bacteria. How bacterial populations respond to selective and co-selective
59 pressures exerted by individual cytotoxic drugs and the implications for antibiotic resistance
60 selection and spread is unknown. Thus, there is an urgent need to understand these potential
61 collateral effects of cancer chemotherapy to ensure effective antibiotic treatment for a large
62 group of immunocompromised patients. Moreover, cytotoxic drugs may constitute a previously
63 unrecognized target for intervention to limit the selection and spread of antibiotic resistance.
64
65 Methotrexate (MTX) is widely used in treatments including but not limited to; cancer
66 of the breast, skin, head, neck, and lung as well as many inflammatory diseases, such as
67 rheumatoid arthritis[9]. We specifically targeted resistance towards trimethoprim (TMP), since
68 both drugs act through inhibiting the dihydrofolate reductase enzyme in bacteria and eukaryotic
69 cells, central in DNA synthesis[10]. TMP in combination with sulfamethoxazole is among the
70 most frequently used antibiotics in the treatment of urinary tract infections and is recommended
71 as first line treatment internationally[11]. Our target organism in this study is *Escherichia coli*,
72 the most common agent of nosocomial infections world-wide[12]. *E. coli* is known to display
73 intrinsic resistance towards methotrexate through AcrAB-TolC mediated efflux[13], however
74 TMP is not a substrate for this efflux system.
75 Previous studies have focused on the abilities of MTX and other non-antibiotics to
76 inhibit bacterial growth[8]. These approaches have provided valuable insights on the effects of
77 non-antibiotics as modulators of the intestinal flora but lacked the necessary resolution to detect
78 more subtle selective effects on acquired antibiotic resistance determinants.
79 Here, we hypothesize that despite the demonstrated *E. coli* intrinsic MTX resistance[13],
80 MTX can affect antibiotic resistance evolution in *E. coli,* due to the shared molecular target
81 with TMP. We show that MTX selects for acquired bacterial TMP resistance (TMP^R) and co-
82 selects for other antibiotic resistance determinants when co-residing on a mobile genetic
83 element. Exposure to a wide concentration range of MTX selects for mutations identical to
84 those emerging during TMP selection in clinical isolates of *E. coli*. Moreover, we show that the
85 minimum selective concentrations (MSCs) of MTX and positive selection for chromosomal
86 and plasmid-mediated TMP^R determinants occurs at concentrations 40- and >320-fold below
87 the MTX minimum inhibitory concentrations (MICs), respectively.
88
89 **Methods**
90 **Bacterial strains and growth conditions:** Bacterial strains used in this study and their
91 assigned reference numbers are listed in Table S1. Most strain constructs were derived from

92 the *E. coli* K-12 strain MG1655 (DA4201/DA5438), or for cloning purposes from *E. coli* DH5α
93 (MP18-09). Two isogenic pairs of clinical *E. coli* strains, where one strain is trimethoprim
94 susceptible (TMP^S) and the other is TMP^R, were used for fitness measurements. In the first pair,
95 TMP^R in *E. coli* K56-2 (MP06-01) is caused by one intragenic point mutation T>A (W30R) in
96 the *folA* gene, and one in its promotor region (P_{folA}, C>T 58 base pairs (bp) upstream of the
97 gene)[14,15] (MP06-05). In the second pair, *E. coli* K56-75 (MP06-41) harbouring the pG06-VIM-
98 1 multi-drug resistance plasmid represents the TMP^R counterpart (MP05-31). This plasmid
99 encodes, amongst seven other antibiotic resistance genes, two different *dfrA* genes (*dfrA1* and
100 *dfrA12*) as putative TMP^R determinants as well as a carbapenem resistance determinant (bla_{VIM-1})[16]. For accurate MSC measurements we constructed pairs of fluorescently tagged (*yfp* and
102 *bfp*) *E. coli* MG1655 strains containing the same resistance markers as described above. For
103 details concerning growth conditions, susceptibility testing and strain constructions see
104 protocols in supplementary information (SI).
105 **Fitness measurements:** Growth rates were determined using a BioscreenC MBR reader (Oy
106 Growth Curves Ab, Ltd). The growth rate calculations were done using the Bioscreen Analysis
107 Tool BAT 2.1[17] (see SI for detailed protocol). Competition experiments were performed in six
108 biological replicates, including dye swaps, using the fluorescently tagged strain pairs described
109 above, containing *folA* or pG06-VIM-1 mediated TMP^R. Both competition experiments, MSC
110 calculations and competitions performed at different starting ratios were done as previously
111 described in[18] (see SI for detailed protocols).
112 **Experimental evolution:** To examine the effect of MTX presence on TMP^R evolution, strain
113 K56-2 (MP06-01) was serially passaged in liquid cultures with MTX supplemented at a
114 concentration slightly above the estimated MSC. Initially, ten independent overnight cultures
115 were started from independent colonies on separate agar plates. From the original overnight
116 cultures, ~10^3 cells were used to start ten independent lineages in 1 mL batch cultures
117 containing 400 μg/mL MTX (lineages 1-10). Every 12 h for 25 days, the lineages were serially
118 passaged by 1,000-fold dilution, allowing for ~500 generations of growth. Every ~50
119 generations the populations were frozen down at –80°C for downstream analysis. For detailed
120 protocols concerning experimental evolution, selective plating on high concentrations of MTX
121 and whole genome sequencing see SI.
122
123 **Results**
124 **Methotrexate selects for pre-existing trimethoprim resistance determinants**

125 We determined the MICs of MTX in clinical and laboratory strains of *E. coli* (Table S1). Initial
126 experiments revealed variable, but high MTX MICs (Table S2) consistent with previous reports
127 demonstrating that *E. coli* displays intrinsic resistance towards MTX due to AcrAB-TolC
128 mediated efflux[8,13]. We also observed that the MTX MIC was dependent on the presence of
129 TMP^R determinants. All isolates with a functional TMP resistance determinant and increased
130 TMP MIC showed consistently higher MTX MICs (>32 mg/mL) than TMP susceptible isolates
131 (4-32 mg/mL), indicating possible co-selective abilities of the two drugs.
132 Antibiotic resistance selection and co-selection have traditionally been assumed to
133 occur between the MICs of susceptible and resistant isolates within a bacterial population
134 (known as the selective window)[18]. However, several reports unequivocally show that antibiotic
135 resistance selection and co-selection can occur at concentrations several hundredfold below the
136 MIC of a susceptible isolate (known as sub-MIC)[18-20]. To test how sub-MICs of MTX affect
137 bacterial fitness, we measured exponential growth rates for two pairs of clinical isogenic TMP^R
138 and TMP^S *E. coli* across a wide MTX concentration span. One pair with TMP^R located on the
139 chromosome (two mutations associated with *folA*)[14] and one pair with TMP^R (*dfrA*) located on
140 the MDR plasmid pG06-VIM-1[16]. TMP^S strains displayed sharply declining growth rates
141 between 1 and 2 mg/mL of MTX, whereas the TMP^R strains remained unaffected (Figure S1).
142 These results suggest a selective benefit during MTX exposure for TMP^R strains at
143 concentrations below the observed MTX MIC of the TMP^S clinical isolates.
144 The MSC describes pharmacodynamically the lowest concentration where selection for
145 resistance occurs[18]. To determine the MSC for MTX, we constructed a fluorescently tagged
146 pair of *E. coli* MG1655 strains to enable accurate separation between the two in mixed
147 populations. In these backgrounds, we introduced TMP^R, either through SNPs (*folA*) using
148 genome engineering or through the pG06-VIM-1 plasmid. The isogenic TMP^R and TMP^S strain
149 pairs were competed head-to-head by serial passage for 30 generations and the ratio of
150 TMP^R:TMP^S was determined over time using flow cytometry. From this data the MSC was
151 estimated (Tables S3-S5)[18]. *E. coli* with chromosomal *folA* mutations had an MSC of 200
152 μg/mL (1/40 of the MIC of MTX) whereas those harboring the plasmid were close to the
153 detection limit of the assay[18] and we conservatively estimated the MSC to be <25 μg/mL (less
154 than 1/320 of the MIC of MTX) (Figure 1). Taken together, our data strongly suggest that
155 selection for TMP^R occurs at MTX concentrations far below the estimated MTX MIC.
156

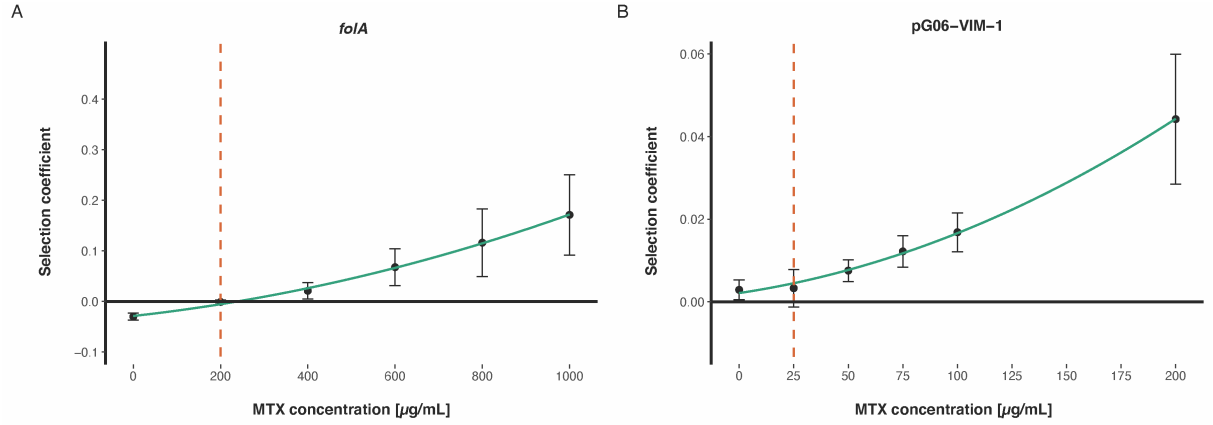


Figure 1: Selection coefficients as functions of MTX concentrations from competition experiments between TMP^R and TMP^S isogenic strains. The MSC is defined as the concentration where the selection coefficient equals zero. The MSC of *E. coli* MG1655 harboring **(A)** two chromosomal *folA* mutations (MP18-04 and MP18-07) is set to 200 µg/mL, and **(B)** the MDR pG06-VIM-1 plasmid encoding *dfrA12* (MP18-05 and MP18-08) is conservatively set at 25 µg/mL. Dashed lines represent the set MSC, bullets the average selection coefficients and error bars the standard deviations.

Sub-MICs of methotrexate promotes invasion of trimethoprim resistance determinants even when rare in *E. coli* populations

Exploring MTX-selective dynamics further, we asked if TMP^R determinants could invade the population at lower initial densities to exclude potential bias from the 1:1 ratio in the competition experiments. We started competition experiments from frequencies as low as 10^{-4} of the TMP^R strains, at concentrations slightly above the estimated MSC of MTX (400 µg/mL for *folA* mediated resistance and 75 µg/mL for pG06-VIM-1 mediated resistance) and followed the change in ratios over 30 generations of growth (Figure 2). Both chromosomal and plasmid mediated TMP^R determinants were able to invade, even when initially rare in their respective populations, strongly suggesting that the MTX selective effects are independent on initial frequencies of resistant and susceptible strains during competition experiments.

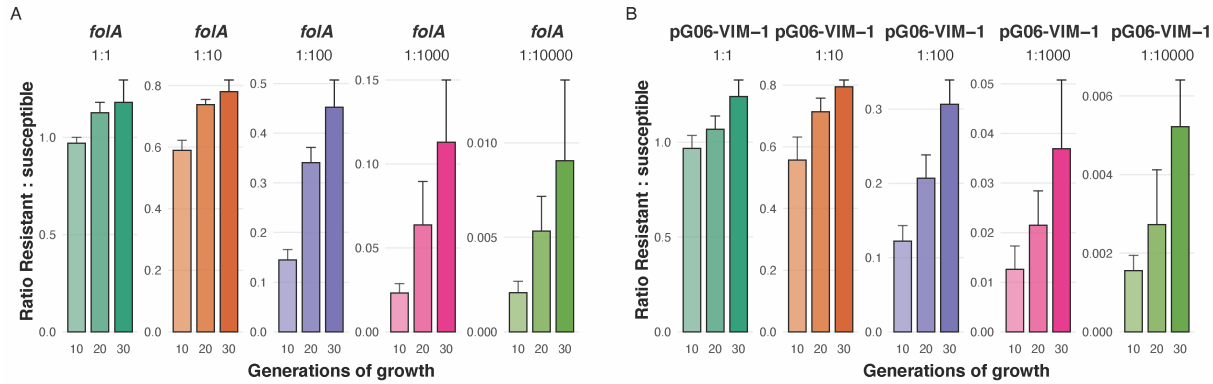


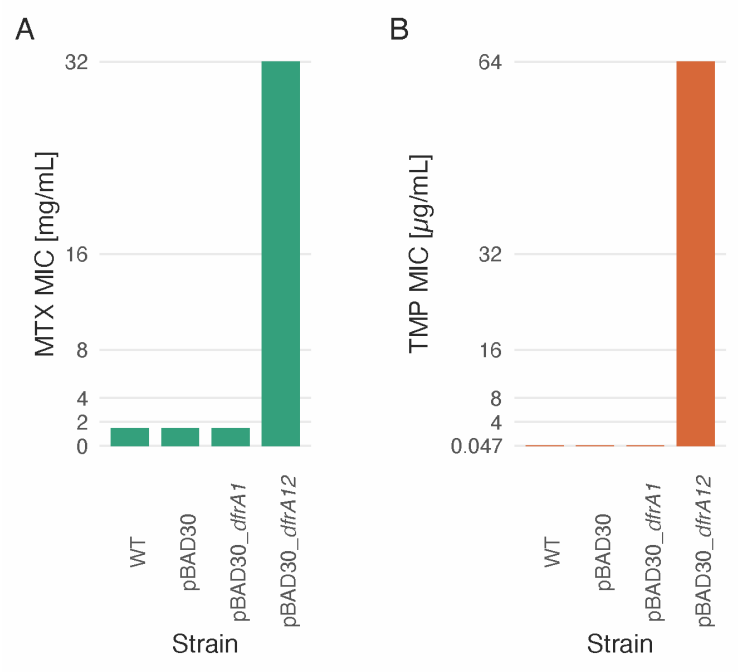
Figure 2: Competition experiments during sub-MIC MXT exposure at different initial frequencies of TMP^R strains. The change in TMP^R:TMP^S ratios over 30 generations of growth at MTX concentrations slightly above the MSCs where *E. coli* MG1655 harbors **(A)** two *folA* mutations (MP18-07), and **(B)** the pG06-VIM-1 plasmid (MP18-08) were competed against a differently tagged, isogenic susceptible strain (DA56507) at 1:1, 1:10^{-2}, 1:10^{-3} and 1:10^{-4} starting ratios. Error bars represent the standard deviation of the average ratio.

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

The *dfr*-genes represent a common TMP^R mechanism in *E. coli* and these genes are frequently located on mobile genetic elements such as integrons and plasmids. Given that MTX selects for *dfr*-mediated TMP^R, co-selection of other genetically linked resistance genes is likely. To show this, we used the MDR pG06-VIM-1 plasmid harboring *dfrA1* and *dfrA12* along with multiple resistance determinants including four aminoglycoside resistance genes and the $bla_{\text{VIM-1}}$ carbapenemase gene conferring resistance to broad-spectrum β-lactams including carbapenems[16]. To assess the stability of pG06-VIM-1 in our strains competing in the presence of MTX, *E. coli* K56-75 harboring the plasmid (MP05-31) was serially passaged in batch cultures with 400 µg/mL MTX supplemented for 50 generations. The lineages were then plated on non-selective agar and 100 colonies from each lineage tested for reduced susceptibility towards ampicillin, TMP, streptomycin and spectinomycin. The results revealed complete phenotypic stability across all three lineages, confirming MTX mediated co-selection of plasmid mediated MDR.

To verify that the TMP^R determinants on the MDR pG06-VIM-1 plasmid is the primary mediators of MTX resistance and selection (Table S2), both *dfrA1* and *dfrA12* were isolated from the plasmid and cloned onto an expression vector and the effects of the individual genes measured. Of the two genes, only *dfrA12* was shown to give the same resistance pattern for

204 TMP as well as MTX as the pG06-VIM-1 plasmid (Figure 3), and the lack of detectable
205 phenotype for *dfrA1* (MP18-11) is likely due to a start codon frameshift mutation[16].
206



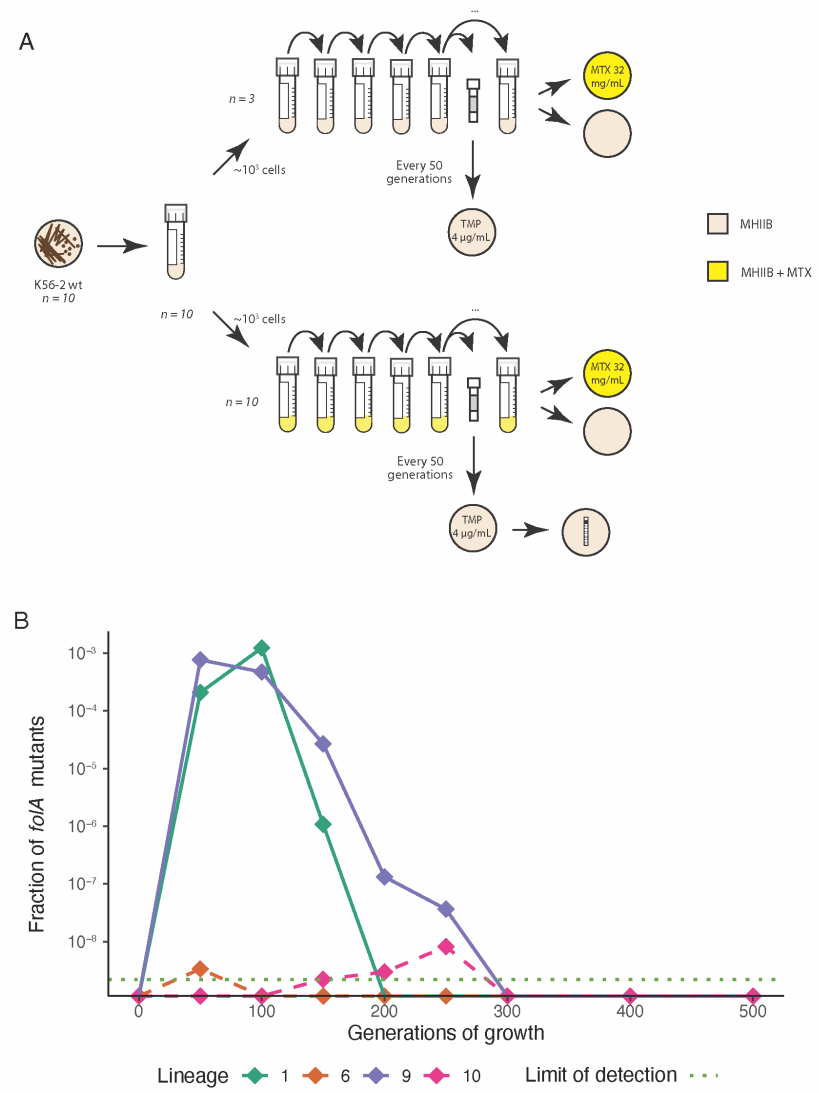
207

208 **Figure 3: MTX and TMP MIC of *E. coli* DH5α expressing *dfrA1* or *dfrA12.*** The MTX **(A)**
209 and TMP **(B)** MIC for the wild type (WT) *E. coli* DH5α (MP18-09) compared to the strain
210 harboring the empty pBAD30 (MP18-10) expression vector as well as strains with pBAD30
211 with different *dfrA* genes expressed under the inducible expression control by the pBAD
212 promotor (MP18-11 and MP18-12). The detection limit of the assay is 64 µg/mL for TMP and
213 32 mg/mL for MTX. For both drugs, the MIC of *E. coli* DH5α expressing *dfrA*12 (MP18-12)
214 exceeded the detection limit whereas the strain expressing *dfrA1* (MP18-11) has the same MICs
215 as the WT strain. Showing that MTX and TMP resistance conferred by the pG06-VIM-1
216 plasmid is caused by the *dfrA12* gene.
217

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

219 We further examined whether exposure to MTX could lead to *de novo* TMP^R evolution. We
220 selected spontaneous mutants from overnight cultures with and without exposure to MTX,
221 plated on selective agar at high MTX concentrations and tested for TMP cross-resistance
222 (Figure S2, Table S6). *E. coli* K56-2 isolated at 16 and 32 mg/mL MTX (MP18-17 to MP18-
223 28) displayed increased MICs of TMP close to or above the clinical breakpoint[21], clearly
224 demonstrating selection for TMP^R by MTX. DNA sequencing of the resistant isolates revealed

225 two different mutations in the *folA* promoter, previously reported to result in TMP^{R22}, as well
226 as a single mutation in the *marR* gene (Table S6-S11).
227 Finally, we asked if exposure to sub-MICs of MTX close to the estimated MSCs would
228 select for *de novo* TMP^{R} mutations in a susceptible *E. coli* population. Starting from 1,000 cells
229 to minimize the probability of pre-existing mutants, we grew ten independent lineages of the
230 *E. coli* K56-2 strain at 400 µg/mL MTX for 500 generations. The frequency of TMP^{R} was
231 determined every 50 generations. TMP^{R} ascended in frequency in 2/10 lineages at different
232 rates and time-points during the first 250 generations before they were outcompeted by a
233 different set of mutants with reduced susceptibility to MTX and no cross-resistance to TMP
234 (Figure 4, Table S12). These experiments show that MTX exposure can select for *de novo*
235 TMP^{R}, both at high and sub-MIC concentrations.
236



237

238 **Figure 4: Evolution of TMP^R during MTX exposure for 500 generations. (A)** Sub-MIC
239 evolution experimental set-up. Ten biological replicates of K56-2 (MP06-01) were evolved for
240 ~500 generations with 400 µg/mL MTX and three biological replicates without drug. All
241 lineages were screened for TMP^R every 50 generations. After 500 generations all end-point
242 populations were plated on 32 mg/mL MTX. All populations were able to grow at 32 mg/mL
243 MTX, but not a single clone isolated conferred TMP^R, strongly suggesting that reduced
244 susceptibility to MTX with no cross-resistance to TMP evolved in the endpoint populations.
245 **(B)** Fractions of TMP^R *folA* mutants isolated every 50 generations from the lineages where
246 these were detected. The detection limit of the assay was ~$2x10^{-9}$. Solid lines represent the two
247 lineages where TMP^R emerged and ascended in frequency whereas dotted lines indicate
248 spontaneous mutants.
249
250 **Pharmacokinetic approximations**
251 To assess pharmacokinetic relevance, we attempted to estimate the MTX concentration range
252 likely to be found in the intestine of patients undergoing MTX treatment. Limited information
253 is available on gut MTX concentrations following intravenous administration during cancer
254 treatment, as pointed out by others[8]. Pharmacokinetic data reveal that up to 90% of administered
255 MTX is renally excreted[23] and we assume that the remaining ~10% of the dose constitutes the
256 upper limit of the concentration range present in the human intestine. The lower limit is set to
257 2% of the dose based on the mean ^{3}H labelled MTX concentrations measured in stool samples
258 from nine patients receiving MTX intravenously[24]. From this, we set a 24 hour transition time
259 in a total volume of 0.6 L [8] and calculated the dose (*d*) required to achieve MSC in the human
260 intestine from:

261 $$\frac{d}{0.6L}\ x\ (0.1\ or\ 0{,}02) = MSC$$

262 Estimated doses needed to reach intestinal MSCs assuming 2% and 10% fecal MTX
263 concentrations were from 0.15g to 0.75g for plasmid-mediated TMP^R and from 1.2g to 6g for
264 chromosomal *folA* mutations. Thus, assuming close to 2m^2 body surface in grown-up patients[25]
265 estimates of MSC for plasmid-mediated TMP^R translates to dosing regimens from 75-375
266 mg/m^2 and from 0.6-3 g/m^2 for the chromosomal *folA* mutations. These approximations indicate
267 that our MSC estimates are relevant for patients receiving high dose MTX treatment (1-12
268 g/m^2)[26]. A recent study used a different approach estimating gut MTX concentrations following
269 oral administration during treatment of rheumatoid arthritis[27]. Their data suggested MTX
270 concentrations as high as 100 µg/mL are found in the gut, suggesting that our estimated MSC

271 for plasmid-mediated antibiotic resistance determinants (25 μg/mL) is well within this
272 concentration range.
273
274 **Discussion**
275 Here we show that exposure to the cytotoxic drug MTX affects selection and evolution of TMP^R
276 determinants at clinically relevant concentrations. Notably, MTX can mediate selection of any
277 antibiotic resistance determinant when TMP^R is co-localized on a mobile genetic element across
278 a wide concentration gradient. Arguably, this potentially important side-effect of MTX
279 treatment has been previously unrecognized, as studies on the effects of non-antibiotic drugs,
280 including MTX, have either focused on bacterial growth inhibition or used drug concentrations
281 around the MIC[8,28-30], with a few exceptions[19,31]. Using the approaches outlined here, including
282 high resolution mixed culture competition experiments, allow determination of the true MTX
283 selective window ranging from the MSC to the MIC[18]. This is particularly relevant for non-
284 antibiotics for which bacteria display reduced susceptibility. In *E. coli,* MTX is a substrate for
285 the AcrAB-TolC efflux pump[13] and selective effects as those demonstrated here would not have
286 been detected in classical susceptibility and/or growth assays in bacterial monocultures. This
287 was recently supported in an *E. coli* chemical genetic screen where clear growth inhibitory
288 effects of MTX, as well as for a range of other non-antibiotics, were only demonstrated in a
289 *tolC* knock-out mutant (i.e. in a mutant lacking the intrinsic mechanism of resistance)[8].
290 Given that many cytotoxic drugs are structurally similar to antibiotics (e.g.
291 doxorubicin/tetracyclines), or target similar key processes as the major antibiotic groups (e.g.
292 DNA/protein synthesis) it is possible that cancer chemotherapy may lead to increased levels of
293 antibiotic resistance in a vulnerable patient group that very often rely on efficient antibiotic
294 treatment for survival. To acquire a deeper understanding of the evolutionary potential of novel,
295 non-antibiotic drivers of antibiotic resistance the approaches presented here are essential. These
296 approaches need to be combined with an improved understanding of the intestinal
297 pharmacokinetics of MTX and other cytotoxic drugs, possible effects of co-administered drugs
298 such as leucovorin mediated MTX rescue[32], and their interactions with the human microbiome.
299 Such knowledge could allow identification of antibiotic + non-antibiotic drug combinations
300 that should be avoided to preempt resistance evolution.
301 Recent studies have showed that non-antibiotics can increase mutation rates[6,7] and
302 promote horizontal gene transfer[31,33]. Taken together with the data presented here it is clear that
303 non-antibiotic drugs have the potential to affect the evolution, selection, and spread of antibiotic
304 resistance determinants. It is also clear that without a better understanding of these potential

305 drivers of antibiotic resistance, interventions including those targeting human consumption of
306 antibiotics may be negatively affected.
307
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318 CIMK. WGS analysis was done using a bioinformatic pipeline designed by JH. WGS data was
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320 and data visualization was done by JSG. The first draft of the manuscript was written by JSG
321 and revised by PJJ. All authors revised subsequent version and provided key edits to the
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323
324 **Competing Interests:** The authors declare no competing interests. **Data availability:** WGS
325 data are available at NCBI (BioProject PRJNA677979).
326
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