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Effect of aspirin on tumour cell colony formation and evolution

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Aspirin is known to reduce the risk of colorectal cancer (CRC) incidence, but the underlying mechanisms are not fully understood. In a previous study, we quantified the *in vitro* growth kinetics of different CRC tumour cell lines treated with varying doses of aspirin, measuring the rate of cell division and cell death. Here, we use these measured parameters to calculate the chances of successful clonal expansion and to determine the evolutionary potential of the tumour cell lines in the presence and absence of aspirin. The calculations indicate that aspirin increases the probability that a single tumour cell fails to clonally expand. Further, calculations suggest that aspirin increases the evolutionary potential of an expanding tumour cell colony. An aspirin-treated tumour cell population is predicted to result in the accumulation of more mutations (and is thus more virulent and more difficult to treat) than a cell population of the same size that grew without aspirin. This indicates a potential trade-off between delaying the onset of cancer and increasing its evolutionary potential through chemoprevention. Further work needs to investigate to what extent these findings apply to *in vivo* settings, and to what degree they contribute to the epidemiologically documented aspirin-mediated protection.

1. Introduction

An important aspect in the fight against cancer is the prevention of disease. While environmental factors, diet and lifestyle changes can modulate the risk of developing cancer [1], emphasis is also placed on chemoprevention, i.e. the regular use of pharmaceuticals that can reduce cancer incidence [2]. This can be an important strategy, especially in patients that are genetically predisposed to certain cancers. A prominent example of chemoprevention is the use of aspirin [3]. Aspirin administration has been shown to reduce the incidence in a variety of cancers [4–8], and has been especially investigated in the context of colorectal cancer (CRC) [9–15]. It reduces the incidence of sporadic CRCs, as well as disease incidence in patients with Lynch syndrome, a genetic predisposition to CRC that involves a mutation in DNA mismatch repair (MMR) genes. While epidemiological data clearly document beneficial effects of aspirin for cancer prevention, the mechanisms underlying this effect are not well understood [16,17]. Aspirin is an anti-inflammatory drug, and inflammation is known to promote the development of a variety of cancers [18], including CRC [19]. It is likely that an important part of aspirin-mediated chemoprevention occurs through interference with these mechanisms. Apart from that, however, aspirin has been shown to exert direct negative effects on cancer cells themselves, which could slow down the rate of early clonal expansion of cancer cells and hence delay the onset of detectable disease [16]. The effect of aspirin on cancer cells can occur through COX-dependent and independent mechanisms, and the exact molecular events responsible remain to be fully understood [16,17].

In previous work, we conducted experiments on CRC cell lines to assess their proliferation and death rates under different aspirin dosage levels [20]. This analysis indicated that aspirin could reduce the rate of cell division and increase the rate of cell death, thus leading to a reduced overall growth rate. While the net growth rate of cancer cells is clearly reduced, these parameters can also affect the rate of tumour cell evolution, especially the rate at which mutants can accumulate. The current paper uses mathematical approaches to investigate how the measured parameter changes brought about by aspirin influence the rate at which tumour cell colonies become established and the rate at which an expanding tumour cell population accumulates mutations. We find that while aspirin reduces the chances that freshly generated tumour cells give rise to an expanding colony, aspirin also increases the chances that mutants have accumulated when a tumour cell population has grown to a given size during aspirin treatment. This suggests the existence of a trade-off.

2. Summary of experiments and data

A panel of eight CRC cell lines (HCT116, HCT116 + Chr3/5, RKO, SW480, HCT15, Caco2, HT29 and SW48) with known mutational backgrounds [21–23] were grown exponentially *in vitro* over 108 h. HCT116 + Chr3/5 is corrected for MMR deficiency by stable chromosome 3 and 5 transfer and was generated in Dr Koi's laboratory [24]. The numbers of live and dead cells were measured over time, as was the cell cycle distribution of the cells at different time points. This was done both in the absence and in the presence of aspirin, at different aspirin doses (0.5, 1.0, 2.5, 5.0 and 10 mM) over 108 h. A mathematical model was fit to these data in order to measure kinetic parameters for the different cell lines and different treatment doses, as described in reference [20]. For the current study, the important parameters that were measured are the division and death rate of the cells. The estimates are summarized in table 1 and form the basis for the work presented here.

3. Computational model to study colony formation and evolution

To describe clonal expansion and evolutionary dynamics during exponential growth of cells, we consider a stochastic linear birth–death process that corresponds to the experiments outlined above. The computational framework that is summarized here has not been newly developed for this study, but is based on previous publications [25–28]. The models are applied here to derive informative quantities and thus conclusions from the data.

Wild-type cells divide with a rate R and die with a rate D . During division, the cells can receive a point mutation with a probability u . This generates a one-hit mutant, which we assume to be either neutral, i.e. characterized by the same growth parameters as the wild-type, or advantageous/disadvantageous, i.e. having a higher/lower division rate compared to the wild-type. The same division, death and mutation processes are assumed to apply to the one-hit mutant, which can give rise to two-hit mutants, and so on.

Mathematical modelling techniques were used to describe theoretically the evolutionary dynamics of mutations in a

Table 1. Parameters R and D (per hour) for each cell line and aspirin dose.

cell line	dose	R	D
HCT116	0	0.042	0.0021
	0.5	0.043	0.0040
	1	0.038	0.0013
	2.5	0.036	0.0028
	5	0.031	0.0015
	10	0.021	0.0074
HCT116 Chr 3/5	0	0.038	0.0020
	0.5	0.037	0.0026
	1	0.036	0.0023
	2.5	0.034	0.0032
	5	0.027	0.0022
	10	0.016	0.0043
RKO	0	0.042	0.0022
	0.5	0.041	0.0023
	1	0.039	0.0024
	2.5	0.034	0.0022
	5	0.029	0.0012
	10	0.018	0.0055
SW480	0	0.034	0.0025
	0.5	0.032	0.0025
	1	0.031	0.0020
	2.5	0.029	0.0020
	5	0.025	0.0026
	10	0.014	0.0030
HT29	0	0.044	0.0080
	0.5	0.043	0.0068
	1	0.044	0.0060
	2.5	0.039	0.0037
	5	0.028	0.0035
	10	0.016	0.0052
Caco2	0	0.033	0.0041
	0.5	0.033	0.0046
	1	0.031	0.0045
	2.5	0.027	0.0044
	5	0.022	0.0056
	10	0.012	0.0080
HCT15	0	0.053	0.0090
	0.5	0.051	0.0083
	1	0.050	0.0084
	2.5	0.049	0.0129
	5	0.035	0.0096
	10	0.016	0.0083
SW48	0	0.039	0.0153
	0.5	0.037	0.0123
	1	0.039	0.0138
	2.5	0.035	0.0109
	5	0.026	0.0157

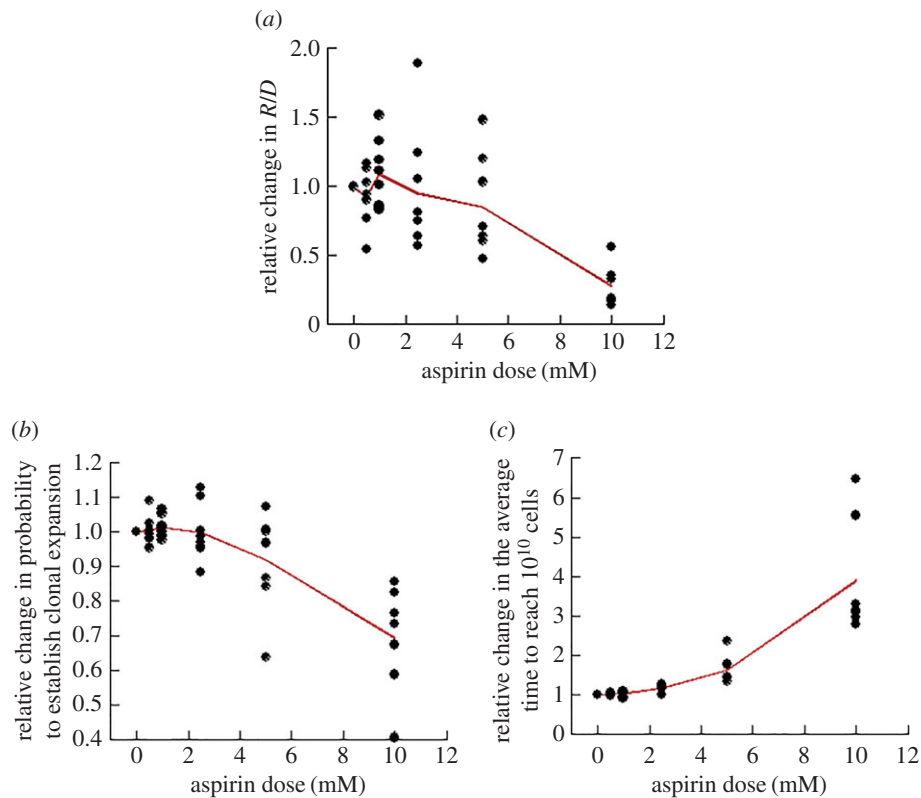


Figure 1. Effect of aspirin on the basic parameters and the dynamics of tumour cell growth: experimental results. (a) Effect on the ratio of the rate of cell division to cell death, R/D . The value of R/D for each aspirin dose is divided by the value in the absence of the drug, yielding the relative change in this measure brought about by aspirin treatment. (b) Effect on the probability for one cell to successfully establish clonal expansion rather than going extinct through stochastic effects, as defined in the text. The graph plots the relative change in the probability to establish growth, brought about by aspirin. That is, the probability to establish growth in the presence of aspirin is divided by the probability in the absence of the drug. (c) Effect on the time it takes for one cell to expand to a population of 10^{10} cells. Again, the relative change is shown, dividing the time in the presence of aspirin by the time in the absence of aspirin. For all graphs, the dots represent the different cell lines under consideration. The line shows the average over all cell lines for each aspirin dose. (Online version in colour.)

colony of cells. We studied a multi-population stochastic birth–death process on a selection–mutation network. A probability generating function was used to calculate the probability that a one- (two-, three-) hit mutant exists at the time when the colony of cells reaches a given size, N . The mean number of mutants at a given size was also calculated. Mutations were assumed to be neutral, advantageous or disadvantageous. Details of the calculations are presented in the electronic supplementary material.

The division and death rates for the different cell lines under the various aspirin doses are taken from table 1. The following investigates how aspirin treatment influences the probability for a single, newly generated tumour cell to give rise to a successfully expanding colony, and how it influences the growth and evolutionary potential of an expanding tumour cell population.

4. Probability to establish a clonally expanding tumour cell population

For now, we do not take into account mutations and simply investigate the probability that a single tumour cell gives rise to an expanding colony of cells under the different aspirin treatment doses. An important basic parameter is the ratio of the cellular division to death rate, R/D , which determines both the growth and the evolutionary dynamics of the cell population. This ratio significantly decreases with aspirin dose (figure 1a). The figure plots the value of R/D for the

different aspirin doses divided by the value in the absence of aspirin, yielding the relative change in this measure induced by the drug. The horizontal axis is the aspirin dose, and the different dots corresponding to the same dose represent the eight different cell lines used. The solid line connects the average values over the eight cell lines for each aspirin dose. In the model, the probability for a single tumour cell to expand and not go extinct is given by $1 - D/R$ (electronic supplementary materials, *1-Prob(Extinct)*, p. 2). This probability decreases with aspirin dose, and the decline is most pronounced for the two highest aspirin doses under consideration (5 and 10 mM, figure 1b). For the highest aspirin dose, the chance for a cell to successfully undergo clonal expansion (rather than to go extinct during this process) is reduced by about 30%. The extent of this reduction, however, decreases with lower aspirin doses (figure 1b). For 5 mM aspirin, the reduction is 3%, and for 2.5 mM it is 2%.

If clonal expansion is successfully established, aspirin can influence the overall growth rate of the tumour cell population. This is illustrated in figure 1c showing how aspirin increases the average time it takes one cell to clonally expand up to a size of 10^{10} cells (corresponding to a detectable tumour). This is given by $\ln(10^{10})/(R - D)$, and normalized relative to time calculated in the absence of aspirin. For the highest dose of 10 mM aspirin, the appearance of a tumour of this size can be delayed on average fourfold. The delaying effect, however, again drops with a reduction in aspirin dose. The delay is 1.6-fold for 5 mM aspirin and only 1.17-fold for a dose of 2.5 mM.

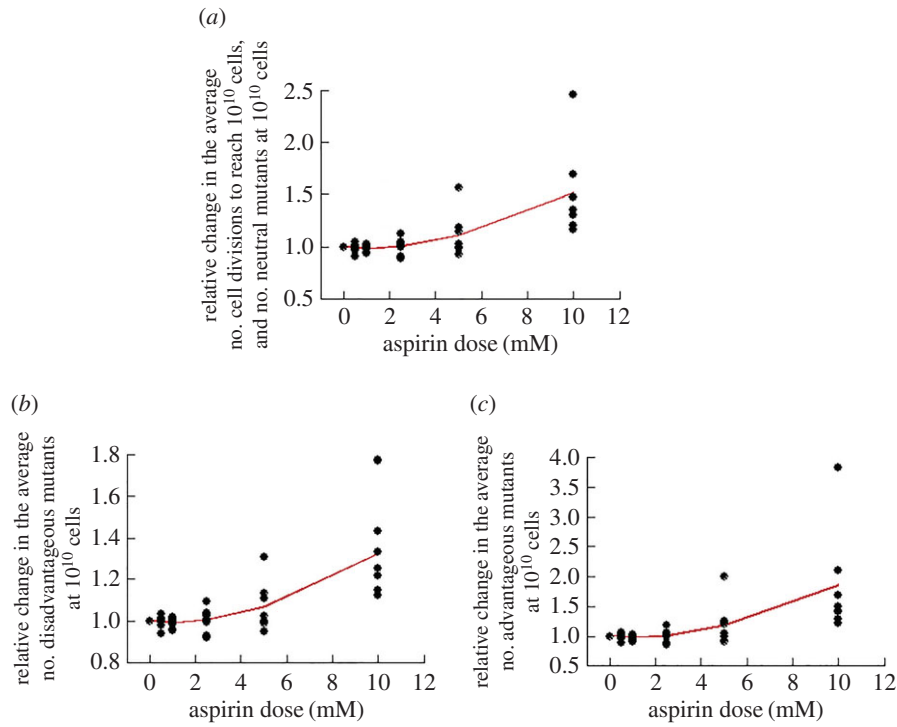


Figure 2. Effect of aspirin on basic evolutionary dynamics. (a) Relative change in the number of cell divisions required to expand from 1 to 10^{10} cells, brought about by aspirin. The number of cell divisions in the presence of aspirin was divided by the number in the absence of the drug. This measure shows the increase in the evolutionary potential of the cell population. The relative aspirin-induced change in the average number of neutral one-hit mutants when the cell colony has reached 10^{10} cells is identical and thus not plotted separately. (b) Relative change in the average number of disadvantageous mutants that are predicted to be present when the cell colony has grown from 1 to 10^{10} cells. (c) Relative change in the average number of advantageous mutants that are predicted to be present when the cell colony has grown from 1 to 10^{10} cells. The dots in the plots correspond to predictions for the different cell lines for each aspirin dose, and the line represents the average over all cell lines for each dose. A mutation rate $u = 10^{-9}$ was assumed. Formulae in electronic supplementary material, S3 were used for this figure. (Online version in colour.)

5. Mutant accumulation and evolutionary potential

An important question is how many mutant cells have been generated during tumour growth by the time the tumour size has reached a detectable threshold, e.g. 10^{10} cells. This depends on the number of cell divisions that are involved to reach this size [26]. The more cell divisions occur, the higher the chance that a mutant is created. Figure 2*a* shows that the number of divisions required to reach 10^{10} cells increases with aspirin dose, especially for the two largest doses. Hence, the mutagenic potential of the tumour can increase with aspirin treatment. This is reflected by the predicted average number of point mutants that have been generated once the tumour has grown to 10^{10} cells. Aspirin increases the average number of mutants, driven by the increased number of cell divisions (figure 2). For neutral mutants, the relative increase in the number of mutant cells is identical to the relative change in the number of cell divisions to reach the population size threshold (figure 2*a*). For disadvantageous mutants, the relative increase in the number of mutants is less pronounced (figure 2*b*), and for advantageous mutants it is more pronounced (figure 2*c*). Hence, aspirin can increase the average number of mutants observed at a certain cell population size, and this increase is larger for a higher relative fitness of the mutant.

Another important evolutionary measure is the probability that a mutant with one, two, three, etc., point mutations is present at the time when the tumour reaches a certain size. This can be important for determining the response of tumours to drug therapies. In several settings, especially in the context

of treatments with small molecule inhibitors, resistance against a drug can be brought about by a single point mutation [30]. In many cases, drug-resistant mutants are thought to pre-exist when treatment is started, with drug therapy leading to the selective outgrowth of these pre-existing mutants [27,29,30]. If only mutants resistant against one drug (e.g. 1-point mutation) are present, a combination of two drugs can potentially avoid treatment failure (in this case, we assume that cross-resistance does not happen, see [31] for the expansion of this theory to the case of cross-resistant mutations [32]). Similarly, if mutants resistant against two drugs (two independent point mutations) pre-exist, but no cell with three simultaneous drug-resistant mutations is present, then a combination of three drugs could potentially prevent treatment failure. While aspirin has no major effect on the probability that one-hit mutants are present at a certain size threshold (figure 3*a*), we observe a significant increase in the chances that two-hit and three-hit mutants are present (figure 3*b,c*). Moreover, the effect is more pronounced for three-hit (figure 3*c*) compared with two-hit mutants (figure 3*b*, see also figure 3*d*). Therefore, aspirin might increase the chances that a tumour that does emerge despite chemoprevention is less treatable if drug combinations are required for successful therapy.

These trends are consistent with basic evolutionary theory. Higher doses of aspirin result in a significant reduction of the ratio R/D . Lower values of R/D are in turn predicted to result in a higher probability that multi-hit mutants exist in the cell population at a defined size, while the chances that one-hit mutants are present are not strongly

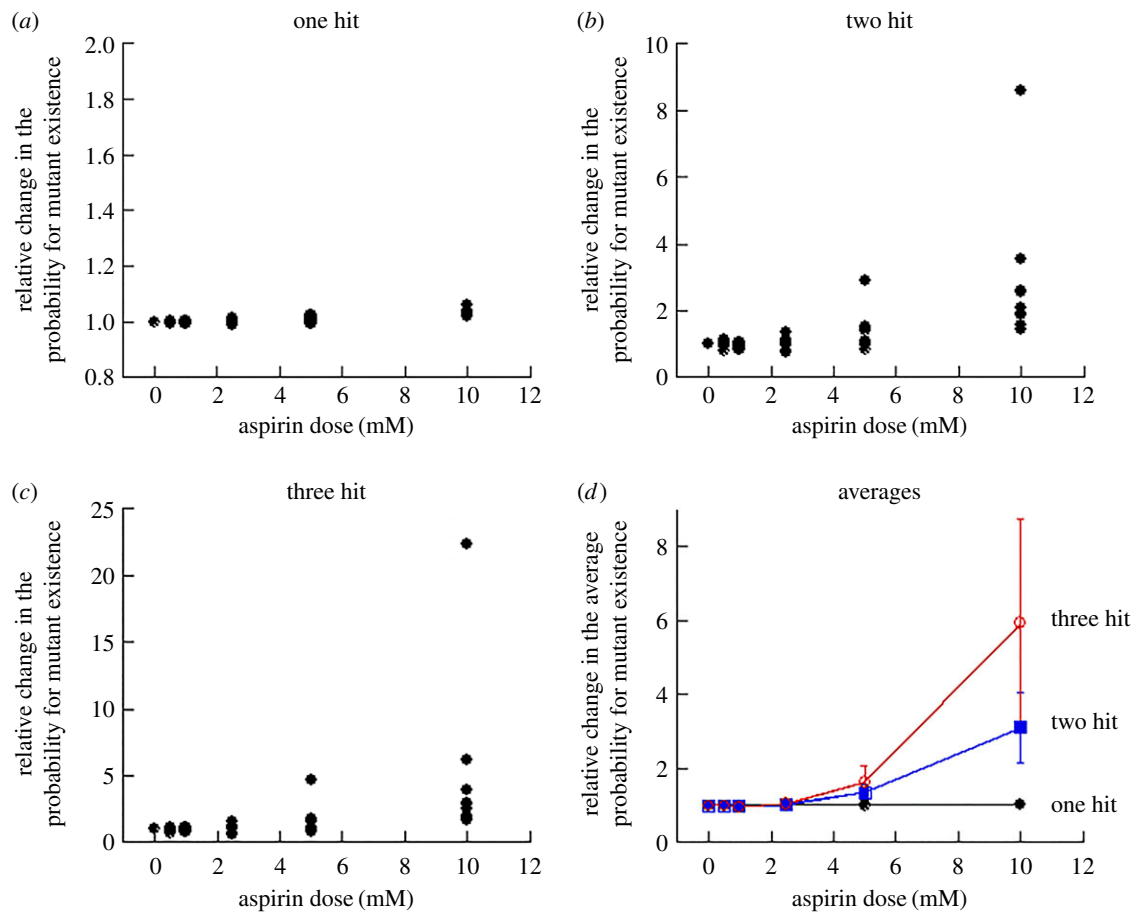


Figure 3. Effect of aspirin on the probability for a cell to exist that is characterized by (a) one, (b) two and (c) three independent neutral mutations by the time a cell colony has grown from 1 to 10^{10} cells (assuming a mutation rate $u = 10^{-9}$). Again, the relative change in the probabilities is shown, dividing the probability for a mutant to exist in the presence of aspirin by the probability in the absence of the drug. The dots represent the different cell lines. (d) This graph plots the average over all cell lines for each dose, along with error bars that represent the standard error. Theory of electronic supplementary material, §§S1 and S2 (and especially formulae (S7) and (S13)) were used to calculate the probabilities. (Online version in colour.)

affected (figure 4). Similar results have been reported in the context of drug resistance against targeted therapy in chronic myeloid leukaemia [27].

6. Discussion and conclusion

Our computational analysis has investigated the effect of aspirin on growth and evolutionary processes in tumour cells, using data on division and death rates from colon cancer cell lines replicating *in vitro*. These data quantified direct effects by aspirin on tumour cell turnover kinetics, which are likely independent from the anti-inflammatory mechanisms exerted by the drug, and should be thought of as one of the aspects of cancer-aspirin interactions. Further, our analysis does not include effects of aspirin on non-transformed cells, which could also contribute to the observed protective effect. The direct effects of aspirin on growing tumour cells, therefore, only represent part of the mechanism that underlies chemoprevention.

Our modelling suggests that aspirin reduces the ratio of R/D , and thus increases the turnover of the tumour cells, which can lead to a reduced probability for a tumour cell clone to successfully expand rather than go extinct. This has the potential to reduce the rate of tumour generation, which in turn could potentially reduce tumour incidence if the initial clonal expansion process results in a detectable lesion (i.e. a tumour of sufficient size to detect by standard tests). If, however, the first clonal expansion process does

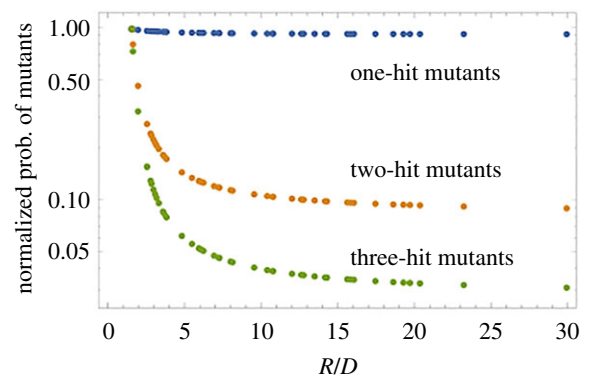


Figure 4. Normalized probability that one-, two- and three-hit mutants are present at a population size of 10^{10} , as a function of the ratio R/D , as predicted by the basic birth–death process considered in this paper (assuming a mutation rate $u = 10^{-9}$). To stay within realistic parameters, this was calculated for the values of R and D in table 1. Lower values of R/D result in a higher probability that a mutant is present, and this effect becomes stronger for a higher number of mutations (two- and three hit). To compare how steeply the probabilities drop as R/D increases in the three cases, we have normalized these functions such that they all start at 1 for the lowest value of R/D . The non-normalized probabilities have vastly different orders of magnitude. Electronic supplementary material, formulae (S7) and (S13) were used to calculate the probabilities. (Online version in colour.)

not result in a detectable lesion, and if further mutations and further phases of clonal expansion are required for that, then the connection of our result to tumour incidence

is more complex. While the connection between the generation (and non-extinction) of the first malignant clone and cancer incidence is not straightforward, the two are definitely connected, and in some cases one can argue that generation/non-extinction is the defining component of incidence. This might be most relevant with Lynch syndrome due to the high mutagenic potential of microsatellite-unstable cells that are generated rapidly in these patients. Microsatellite-unstable cells generate a substantially greater number of mutations due to the loss of DNA MMR [31], a process that appears to be highly immunogenic, and contributes to the more favourable natural history of hypermutable colorectal cancers [32,33]. Thus, the generation of additional mutations may be relatively less important in the context of a hypermutable milieu.

If a tumour cell clone does expand successfully in the presence of aspirin, our calculations indicate that such a tumour cell population can have an increased evolutionary potential, especially with respect to the emergence of mutants with two or more hits, which could correspond to a more aggressive cell clone or to simultaneous resistance against more than one drug. This result indicates that there could be a trade-off between chemoprevention and the evolutionary potential of the tumour. That is, while aspirin is predicted to reduce the chances that malignant cells successfully expand, if a tumour does grow during aspirin administration, this tumour will have accumulated more mutations at a given size compared with a tumour that grew in the absence of aspirin. If this is the case, it will be critical to make sure that aspirin-based chemoprevention efforts delay the onset of CRC by a sufficient amount of time to avoid the negative effects of this trade-off, or that people who take aspirin are regularly screened for early-stage CRC. If an emerging tumour is not detected and does grow to larger levels during aspirin treatment, it might be more difficult to manage therapeutically due to the increased mutational load. We note that this is a modelling insight and has so far not been investigated in the context of epidemiological data. Regular use of aspirin after diagnosis has been associated with a lower risk of CRC-induced mortality in the context of primary tumours with COX-2 overexpression, which is likely due to the negative effect of the drug on the clonal expansion processes documented in our study. Whether the trade-off predicted here can be observed in epidemiological data depends on how treatable to cancer is once it has progressed to a larger size. If treatment success is limited in such situations, it might not matter for survival whether the cancer has accumulated more or less mutations by the time this size-threshold has been reached.

It is important to point out that our results are based on the response of cell lines *in vitro* and that *in vivo* responses to aspirin could be different. Furthermore, the most pronounced impacts of aspirin in our experiments were observed with the highest aspirin doses (5 mM and 10 mM), which are above the concentrations that are achieved physiologically. In fact, the maximum tolerated concentration in patients is thought

to be 2 mM [33]. While the same general trends were found for lower concentrations, the effects were significantly less pronounced. On the one hand, it can thus be argued that the direct effect of aspirin on tumour cell kinetics is limited. On the other hand, it is also possible that even subtle effects that are operating over a relatively long period of time can amount to a significant protective effect that can be reflected in epidemiological data. In addition it is clear that the *in vivo* environment is significantly different from the *in vitro* conditions characteristic of our experimental set-up. While in our *in vitro* set-up, exaggerated aspirin doses are required to see relatively strong effects, microenvironmental effects *in vivo* might result in a situation where these effects are amplified at physiological aspirin concentrations. Given the observations reported here, further work is required with *in vivo* systems, such as cell-line derived mouse xenografts or patient-derived xenografts.

In general, however, available data paint a clear picture suggesting that any potential direct effect of aspirin on tumour cell kinetics can only be part of a host of mechanisms that contribute to aspirin-mediated chemoprevention. Aspirin likely influences evolutionary processes in healthy tissue that precede the generation of tumour cells and that delay their generation. In epidemiological data, the power of aspirin-induced CRC prevention depends on the patient cohort under consideration and on the protocol of aspirin administration. Significant reduction in sporadic CRC incidence among individuals with average risk has been reported, which is a function of aspirin dose and the duration of treatment [13,14]. Overall, among individuals taking aspirin for 10 or more years, a 21–23% reduction of incidence has been observed. The exact magnitude, however, depended on dose, with up to a 70% reduction in incidence for the highest doses considered (greater than or equal to 14 325 mg aspirin tablets per week) [13,14]. A 63% reduction of CRC incidence has been observed among patients that are characterized by Lynch syndrome, a hereditary predisposition to CRC, given 600 mg of aspirin daily for a period of at least 2 years [11]. This result was obtained after an average of 29 months of aspirin administration and a 55-month follow-up. Without the prolonged follow-up, however, aspirin did not significantly reduce the incidence of tumours, which mostly consisted of adenomas at that time point [34]. These observations clearly indicate considerable complexity in the mechanisms that underlie the protective effect of aspirin, and the potential mechanisms considered in our paper are only one aspect that might play a role.

Data accessibility. All relevant data are presented in this paper and in the electronic supplementary information.

Authors' contributions. D.W. designed the study, performed, calculations, ran computer simulations and wrote the paper. A.G. designed the experiments upon which this paper is based, interpreted the data and wrote the paper. C.R.B. designed experiments upon which this paper are based and wrote the paper. N.L.K. designed the modelling approach, performed calculations and wrote the paper.

Competing interests. We declare we have no competing interests.

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