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Genotoxicity of 2-nitro-7-methoxy-naphtho[2,1-b]furan (R7000): A case study with some considerations on nitrofurantoin and nifuroxazide

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Abstract

Two nitrofurans present broad-spectrum antimicrobial properties and some of them are used in human and veterinary medicine. Most of these molecules are mutagens and some of them were reported as carcinogens. Due to its extreme mutagenic potency in bacteria, the nitronaphtho derivative 2-nitro-7-methoxy-naphtho[2,1-b]furan (R7000) was used as a tool to analyze the mechanism of the genotoxic action of this family of chemicals. In the present paper, we review essential data on the genotoxicity of R7000 and briefly discuss the case of nitrofurantoin and nifuroxazide, two nitrofurans, still in use as urinary and gastrointestinal disinfectants. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

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1. Introduction

5-nitrofurans have been widely used as antibacterial and antiparasitic agents, as well as food and feed additives. The fact that most of them are mutagens and some are carcinogens has reduced their usage. For example, furylfuramide (AF2) which was found to be mutagenic in bacterial tests and carcinogenic in animals, was banned as a food additive in Japan in the seventies (see reviews in [5]). However some compounds of the series, such as nitrofurantoin and nifuroxazide, are still used as antibacterial agents in the human.

In the early eighties we examined genotoxicity in the *Salmonella*/microsome assay of a number of benzofurans and naphtofurans, one of which, 2-nitro-7-methoxy-naphtho [2,1-b]furan (R7000) (Fig. 1), was a candidate antiparasitic drug [47]. Due to its extreme mutagenicity, this product was never developed nor marketed. We decided to try to further characterize its genotoxic properties and to elucidate as fully as possible the reasons for its extreme activity. In order to try to relate the genotoxicity in bacteria to the genotoxicity in mammals, we studied the effects of R7000 on mammalian cells in culture [1] and, more recently, on mice transgenic for the *lacI* gene of *Escherichia coli* ("Big Blue Mice") [31].

In the present paper, we review the essential data on the genotoxicity of R7000 and discuss briefly the case of nitrofurantoin and nifuroxazide.

2. Mutagenic and SOS-inducing abilities in bacteria

With one possible exception only 2-nitro-naphtho and benzofuran derivatives gave a mutagenic response in the *Salmonella*/microsome assay [1,46]. The mutagenic response was at least partially dependent on the nitroreductase activities of bacteria. It was in general lower in the presence of an activating mixture from rat liver, suggesting that at least some of the microsome-activated derivatives did not reach the bacterial DNA.

The mutagenic potency depended notably on the nature and position of the other substituents on the molecules (Fig. 1). Starting from nitrofuran (mutagenic potency: 0.26 revertants/nmole on strain TA100), the mutagenic potency of the nitrobenzofuran derivative is about 1000 times higher. The addition of another benzene ring on 2-nitrobenzofuran yields 2-nitronaphthofuran with a further increase in mutagenic potency of a factor about 100. The substitution of a methoxy in position 7 of 2-nitronaphthofuran, yielding 2-nitro-7-methoxy-naphtho[2,1-b]furan (R7000), increases again the mutagenic potency by a factor of about 10. The substitution of a methyl on the heterocyclic

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[1] This review is the last paper that Maurice Hofnung wrote.

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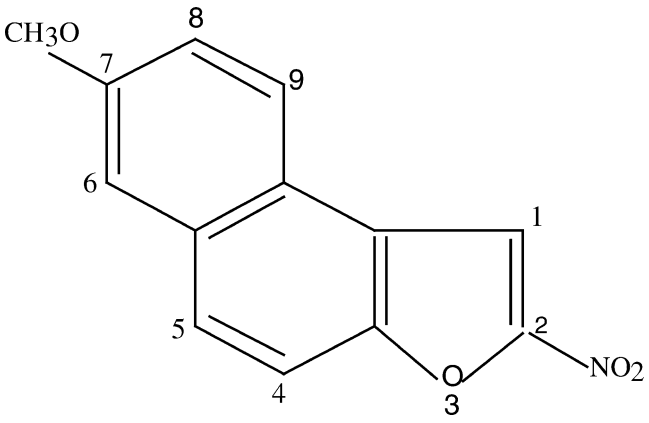

Fig. 1. Structure of 7-methoxy-2-nitronaphtho[2,1-b]furan (R7000).

ring at position 1 results in a supplementary increase by a factor of about 10 yielding 2-nitro-1-methyl-7-methoxy-naphtho[2,1-b]furan (R7372) ($\sim 2 \times 10^6$ mutants/nanomole on strain TA100). Remarkably, the coefficient of increase of the mutagenic potency depends mainly on the substituent and its position, and very little on what has already been substituted elsewhere on the 2-nitrofuran skeleton [1]. The reasons for these multiplicative effects are not known. They could correspond, for example, to modifications in the interactions with DNA, to modifications in the metabolism of the drug leading to activation, or to modifications in the entry of the drug in the bacterial cells.

A series of 2-nitrobenzofurans and 2-nitronaphtofurans were tested for their ability to induce the SOS system in *Escherichia coli* [1]. This was performed in a standard assay called the SOS chromotest [30]. It involves a strain carrying a gene fusion between gene *sfiA* which belongs to the SOS regulon and beta-galactosidase which is monitored as a reflection of induction of the regulon. Interestingly, for all compounds tested there was a good correlation between the SOS inducing potency (SOSIP) and the mutagenic potency, indicating that the nitrofuran derivatives were mostly mutagenic through the action of SOS-dependent genes [46]. This hypothesis is supported by the fact that induction of reversion of the *hisG46* misense mutation is not seen in strain TA1535 but occurs in strain TA100 which carries the plasmid pKM101 [26]. This plasmid is known to carry the *mucA,B* operon which is responsible for SOS mutagenesis in the tester strains of the *Salmonella*/microsome assay [27,45].

3. Bacterial metabolism of nitrofurans

The presence of the NO_2 substituent at position 2 on the furan ring was essential for the genotoxic activity of furan derivatives [1,5]. As for other nitro compounds, nitroreduction is an important step in the metabolic activation of nitrofurans. Accordingly, R7000-resistant bacteria were selected and characterized as nitroreductase-deficent strains [26].

In *E. coli*, two nitroreductases have been reported which were distinguished on the basis of their oxygen sensitivity. The type I enzyme, insensitive to oxygen, consisted of one major component encoded by *nfsA* and two others specified by *nfsB* an unidentified gene [25]. This nitroreductase was genetically characterized through the selection of nitrofurazone-resistant bacteria [25]. The strain deficient for NfsA and NfsB showed an increased resistance to toxic effects of R7000 as compared to the wild-type strain. The total level of R7000-induced DNA adducts characterized by post-labeling analysis as well as the resulting mutagenic potency of the compound are greatly affected in the double mutant *nfsA, nfsB* [Touati, unpublished results]. This indicated that the type I nitroreductase encoded by *nfsA* and *nfsB* is involved in the metabolic activation of R7000, leading to the formation of DNA-damaging metabolites accounting for the mutagenic activity of the compound.

Polycyclic aromatic nitrocompounds can also be reduced in vitro via a chemical mechanism as reported in the case of 1-nitropyrene [12]. In vitro incubation of R7000 with DNA under reducing conditions (ascorbic acid/sodium citrate pH 5) led to the formation of DNA adducts characterized by postlabeling analysis [Quillardet, unpublished results]. Thus, nitroreduction is essential to trigger the metabolization of R7000 resulting in the formation of DNA-damaging derivatives.

4. Adducts to DNA in bacteria

Two different approaches were used to demonstrate DNA binding by metabolic derivatives of R7000 in vivo: (i) measurement of the binding of derivatives of radiolabelled R7000 and (ii) detection by the "^{32}P-postlabelling" method of DNA adducts following treatment with unlabelled mutagen. It was also directly shown that the formation of adducts required metabolic activation [43].

Analysis of the binding to DNA of R7000 tritiated on position 6 (Fig. 1) was performed in an *E. coli* strain carrying a *uvrA* and an *rfa* mutation. *uvrA* prevents the excision of bulky DNA adducts while *rfa*, which affects lipopolysaccharide, allows faster entry of R7000 into the cell. In the conditions used (complete medium, late stationary phase of growth), the number of molecules bound was 3 per 10^6 base pairs of chromosomal DNA for an initial concentration of 0.6 micromoles of R7000 in the medium. No significant differences were found between binding to chromosomal DNA and binding to plasmid DNA. It was estimated that only a small fraction (0.04%) of the initial radioactivity was bound to DNA [43].

The tritiated product was found to induce the SOS system with the same efficiency as the unlabelled compound. In the SOS chromotest, the induction factor was about 20 for a binding of 0.65 molecules per 10^6 base pairs of DNA. The relation between the SOS induction factor and the binding to DNA was quasi-linear at least up to 0.8 molecules per 10^6

base pairs of DNA. For a binding of 0.1 molecule of R7000 derivative per 10^6 base pairs of DNA an SOS induction factor of 2.5 was found. Since the number of base pairs of the *E. coli* chromosome is about 5×10^6 and since, in the conditions used, there are usually 2 chromosomes per cell, this indicates that a single adduct per chromosome is sufficient to appreciably induce the SOS response. This may be one clue to the very high mutagenic potency of R7000, since it suggests that the nature of the damage caused by R7000 is responsible for its high genotoxic activity.

With the ^{32}P-postlabelling method, at least nine nucleotide modifications were detected, two of which accounted for about 50% of the total binding. This result provided additional evidence for the formation of DNA adducts by R7000 in bacteria [43].

5. Target nucleotides in bacteria

Since R7000 forms DNA adducts and since the study of mutagenic specificity by reversion of His$^-$ revealed mainly GC->TA transversions and to a lesser extent AT->TA transversions [32], it is tempting to speculate that the adducts occur mainly at G:C base pairs. In order to determine precisely the target nucleotides involved in the R7000–DNA interaction three different approaches were used.

First, plasmids harboring GC-rich and AT-rich fragments were treated in vivo with R7000 in a *uvrA recA* strain to minimize the effect of DNA repair. Analysis of the GC-rich and AT-rich sequences of the isolated plasmids with the ^{32}P-postlabelling method revealed that the amount of covalent binding was 100 times higher on the GC-rich fragment than on the AT-rich fragment. However, because of the exact nature of the genetic constructions used, it was not possible to exclude a low adduction on AT base pairs. Second, by using a DNA fragment of known sequence and an adaptation of the Maxam-Gilbert chemical method for DNA sequencing, it was shown that R7000–DNA adducts caused alkali-labile lesions specifically at guanine residues. Third, an adaptation of the dideoxynucleotide sequencing method was performed where DNA lesions in the template act as chain terminators. The analysis was performed on supercoiled plasmids extracted from R7000-treated bacteria. The DNA replication was catalyzed by avian myeloblastosis virus (AMV) reverse transcriptase and the reaction products were analyzed by sequencing gel electrophoresis. The positions of elongation arrests were determined by comparison with a lane of dideoxy sequencing reactions using untreated plasmid DNA. AMV reverse transcriptase termination on R7000-treated DNA occurred preferentially at the level of guanines in the template [44].

In conclusion, all the approaches show that adducts occur essentially, if not only, at guanine residues in the DNA of R7000 treated bacteria.

6. Mutagenic specificity in bacteria

A first characterization of the mutagenic specificity of R7000 was performed with a battery of His$^-$ mutants of *Salmonella typhimurium* designed for this purpose [22,32]. It was shown that R7000 could induce a large mutational spectrum. It could, in particular, induce base-pair substitutions, mainly GC->TA transversions and, to a lower extent, AT->TA transversions. R7000 also appeared to be a very efficient inducer of base-pair deletions/insertions resulting in frameshifts. However, whereas the induction of base pair substitutions by R7000 was dependent on plasmid pKM101 carrying the *mucA,B* operon [27], the induction of base-pair deletions/insertions was independent of the plasmid [32].

In order to more accurately assess the mutagenic specificity of R7000 we analyzed the nature and sites of mutations induced in the *lacI* gene of *E. coli* K12. In addition, in order to obtain insight into the mechanisms of mutagenesis and to evaluate the influence of SOS functions, the mutational spectrum obtained in an *umuC*$^+$ strain was compared to that in an isogenic *umuC* context. The *umuD*$^+$*C*$^+$ operon is the only chromosomal SOS encoded locus that must be induced for SOS mutagenesis to occur [4,40].

In both contexts, 80% of all mutations occurred at G:C sites, indicating that most of the premutagenic lesions formed by the mutagenic derivatives of R7000 involve guanine and/or cytosine. This agrees with experiments demonstrating that R7000 adducts occur essentially at G:C base pairs [44]. The few mutations observed at A:T sites could result from minor adduct formation at A:T base pairs or from DNA replication errors caused by adjacent or nearby lesions at G:C base pairs. As noted above R7000 is a very efficient inducer of the SOS functions which may also lead to untargeted mutagenesis.

Independently of the *umuC* allelic state, R7000 induces numerous classes of mutations, mainly substitutions and single (-1) frameshift events. The mutagenic spectrum is complex and more than one type of mutation occurs at several sites. This could be due to the formation of several distinct premutagenic lesions at the same sites; indeed it was found that at least 9 different nucleotide modifications could result from the action of R7000 on bacteria [43]. Another possible reason would be different mode of resolutions of the same lesions, for example by different error prone DNA polymerases [3,13].

In the *umuC*$^+$ strain, the main substitution events were G:C->T:A transversions. The preferential nucleotide sequence in the vicinity of G-substituted bases is 5-′TGG CG-3′. Among 6 such sequences in the *lacI* gene, 5 were mutated in our experiments [42]. The distribution of R7000-induced mutations at guanine sites was: A = 58%, T= 27% and G = 15% almost identical to that found by Kunkel for the incorporation of bases incorporated opposite a non-instructive lesion such as apurinic sites resulting from the loss of guanine [18]. It is thus tempting to speculate that apurinic sites are intermediates in the error

prone repair pathway for the R7000-induced mutations at guanines. This hypothesis is supported by the demonstration of the existence of alkali-labile sites at guanine residues after R7000–DNA interactions in vivo [44]. In the *umuC*-deficient strain the global proportion of substitutions was unchanged as compared to the *umuC*$^+$ strain, but the major event was G:C->C:G transversions instead of G:C->T:A transversions and the nucleotide distribution opposite substituted guanine was: A = 28%, T = 24% and G = 45%. This observation indicates that the specificity of the substitution depends on the *umuC* dependant mutagenic process.

In both cases (*umuC*$^+$ and *umuC*) the vast majority of frameshifts involved the deletion of one G:C base pair. They occurred preferentially at runs of identical bases and their number increased with the length of the run. This is compatible with the early model where the deletion would occur by slippage of the template strand relative to the primer strand during DNA synthesis [41]. This process would be favored by the adduct and dependent on its position within the run [20]. The global frequency of single G:C deletions induced by R7000 was similar in *umuC*$^+$ and *umuC* backgrounds. However, assuming that the single frameshifts at G:C base pairs were due to adducted guanines, it was proposed that there was a distinct influence of the *umuC* product on leading and lagging strands during translesion synthesis [42].

Despite the fact that R7000 is among the most potent inducer of the SOS functions, in the mutagenesis conditions used, there was no marked effect of *umuC* deficiency on the mutation frequency. This leaves open the possibility that a *umuC* independant pathway is implicated in the mutagenic bypass of the non-instructive lesions created by R7000 [32].

7. Replication blocks induced in vivo in the *lacI* gene of *E. coli* by R7000

DNA adducts that block replication, induced in vivo by the 5-nitrofuran derivative R7000 (7-methoxy-2-nitronaphtho[2,1-*b*]furan) were mapped, at nucleotide resolution, in a region of the *lac I* gene of *E. coli*, using a reiterative primer extension assay [6,29]. It was found that R7000 induced a broad spectrum of low frequency replication blocks rather than some hot spots in a limited number of particular targets. Most of these replication blocks were observed at G nucleotides, and most, if not all, of the G nucleotides present in the DNA sequence constituted a possible target for the chemical attack of the compound. In addition, a large number of replication blocks observed at A, C or T could also reflect a replication block at the 3′ or 5′ nucleotide flanking a guanosine-DNA adduct. Only a very small number of replication blocks could be observed at A, C or T nucleotides non adjacent to a G. These results again show that guanosine-DNA adducts are the main DNA lesions that block replication induced by R7000 in *E. coli* and suggest a strong reactivity of the genotoxic species generated in vivo by R7000 with the guanine targets. From 26 R7000-induced mutations previously mapped in this region [42], 22 (85%) occurred at G:C base pairs at which termination products were observed. The other mutagenic events involved A:T base pairs adjacent to a guanine nucleotide forming a replication block. Thus all mutagenic events occurred at, or adjacent to, a guanine nucleotide forming a replication block. Although it could not be excluded that some mutagenic events are due to undetected DNA lesions that do not block replication, these results strongly suggest that guanosine-DNA adducts that block DNA replication are responsible for a large part of the mutagenic events generated by R7000. The powerful capacity of R7000 to form adducts at most guanosine residues in a DNA sequence may account for at least part of its very potent genotoxic properties.

8. Influence of the *uvr*-dependent nucleotide excision repair

Like many chemicals which are able to induce DNA distortion, toxic and genotoxic properties of R7000 are strongly enhanced in bacteria deficient in the nucleotide excision repair system [47]. The influence of this DNA repair system on DNA adduct formation, SOS induction and mutagenic specificity was studied in *E. coli* [33]. Binding of ^{3}H-labelled R7000 to DNA indicated that R7000–DNA adducts can be removed by excision repair soon after the action of the chemical: 50% of the adducts were removed within 10 min of the treatment. After 1 h of incubation, the level of excision reached 70%. This result was confirmed using the postlabelling technique. It was confirmed that R7000 yielded at least 9 different DNA adducts. Each of the adducts detected could be removed by excision repair. The rates of excision appeared different from one another, but the maximum level of excision was relatively similar from one adduct to another since it varied from 88% for the lower to 96% for the highest. These results fit well with the fact that in the presence of R7000, mutagenesis (measured by the appearance of rifampicin-resistant mutants), survival and SOS inducing potency (measured in the SOS chromo test) were 5 times higher in the *uvrA* strain than in the *uvr*$^+$ strain, suggesting that the *uvr*-dependent repair pathway can remove about 80% of R7000-induced DNA lesions which can be processed in the *uvrA* mutant, via some error-prone neutralization pathway.

Using a *lacZ* reversion system that is able to detect each type of base substitution mutation, we found that in *uvrA* bacteria deficient in excision repair, R7000 can induce 5 of the 6 possible mutational events: G:C->T:A, A:T->T:A, G:C->C:G, A:T->C:G and G:C->A:T. The transition A:T->G:C was not observed. Interestingly, only 3 substitutions (transversions) could be detected in repair-proficient *uvr*$^+$ bacteria: G:C->T:A, A:T->T:A and G:C->C:G. The differences between the mutagenic spectra obtained between the *uvr*$^+$ and *uvrA* bacteria indicate that some poten-

tially mutagenic adducts induced by R7000 can be removed by excision repair, thus lowering the mutagenic potency of the chemical and modifying the mutagenic spectrum detected [33].

The influence of the *uvr*-dependent excision nucleotide system upon the mutagenic specificity of R7000 was also recovered in the *lacI* gene of *E. coli*, as previously described (see above). The main difference from the previous mutation spectrum determined in the wild-type genetic context, is that GC->CG, AT->CG and AT->TA occurred in the same proportions among substitution base pairs (24%), as did, to a lesser extent, GC->AT and GC->TA (9%). We should point out the presence of frameshift events such as single addition and double deletion base pairs, which were not previously observed with the Uvr-proficient strain [Lerondeau (1998) DEA in microbiology]. The repair of DNA lesions leading to these mutations was likely to be strictly Uvr-dependent. This mutation spectrum exhibited more diversity than that determined in the wild-type genetic context. In agreement with postlabeling data [33], this result indicated that R7000 induced numerous DNA lesions with various chemical structures, mainly repaired by the Uvr system.

One of the aims of this study was to characterize events in the mechanism of action of R7000 with DNA which are specific to its very high genotoxic potency. For that purpose, a comparison was performed in a *uvrA*-deficient strain with the mutation spectra in the *lacI* gene, induced by another derivative from the 2-nitronaphthofuran series, 2-nitronaphtho[2,1-b]furan (R6597) which is 10 times less mutagenic than R7000, in the *Salmonella*/assay [1]. R6597 induced mainly substitution base pairs, 65% of the events, essentially GC->TA and AT->TA transversions. Single frameshifts represented 25% of the mutations, consisting essentially of deletions of one (G,C) base pair. Except for the high proportion of substitution base pairs, this mutation spectrum is not very different from that induced by R7000 under the same conditions. This suggested that R6597 and R7000 induced chemically similar premutagenic lesions at common target positions, but in the case of R7000, different mutational events were observed for the same site, indicating that multiple DNA lesions can be generated at the same position. In addition, DNA secondary structure seemed to direct the R7000 interaction, since a number of mutations (10% of the mutants in each cases) were clustered in regions 20 to 30 nucleotides long, with complementary nucleotide sequences at 5′ and 3′ sides.

9. Mutagenic properties of R7000 in “Big Blue” transgenic mice

In higher organisms, early and preliminary evidence suggested weak carcinogenic effects of R7000 on a limited number of animals. After i.p. injection of R7000 into rats or mice, malignant tumors were observed only at the site of injection [21,34,35]. After oral administration, tumors appeared in the forestomach, a site where the compound collects after swallowing [36]. Although R7000 induces mutations in cultured mammalian cells [1], no data were available about the mutagenicity of the compounds in mammals in vivo. To obtain such data, we used the Big Blue transgenic mouse mutation detection system that allows direct analysis of in vivo somatic mutations [31,37]. Both the frequency and pattern of spontaneous or induced mutations can be assessed in any tissue or cell type. The Big Blue system [15,16] uses transgenic mice harboring chromosomally integrated lambda bacteriophage containing the *E. coli lacI* gene as a target of mutagenesis. Lambda phages containing the *lacI* gene are retrieved by in vitro packaging from murine DNA. Individual infectious phage particles are screened for mutations by infecting *E. coli* cells and plating the infected cells in the presence of a chromogenic substrate (X-gal) for β-galactosidase: *lacI* mutants give rise to blue plaques on a background of colorless wild-type plaques.

First, the genotoxic properties of R7000 were examined in vivo after intraperitoneal (i.p.) administration in Big Blue mice. The mutation frequency was determined in various organs of treated mice and the nature of the induced mutations was determined in the target organs. It was found that R7000 is mutagenic in mice. The most affected organs, small intestine, caecum and colon organs, belong to the digestive apparatus. The distribution of R7000-induced mutations in the *lacI* gene recovered from the small intestine of transgenic mice was very similar to that which had been found in *E. coli* [42]. The differences between mouse and *E. coli* in the R7000-induced mutational spectra were mainly in the proportion of single base frameshifts versus base substitutions [2,31].

The influence of time was analyzed on R7000-induced DNA adducts detecting by the postlabelling method, their disappearance and the resulting mutation frequencies and nature [2]. The analysis was carried out on two target organs: the small intestine and the caecum. Mice were treated by intraperitoneal (i.p.) injection of one daily dose of R7000 for five consecutive days, and sacrificed at various times, from 3 h to 25 days after the last injection. Relationships were observed between the decrease in R7000–DNA lesions, as judged by postlabelling, and the induction of mutagenesis. The chemical structure of the DNA lesions detected by postlabelling seemed different for the two organs. As seen by postlabelling analysis, in the caecum, R7000 leads to 14 structurally different nucleotide modifications. In the small intestine, a 10-times lower level of adducts as compared to the caecum was seen, and only 7 distinct induced DNA adducts could be observed. Several nitrofurans metabolic pathways are effective in mammals [23]. Enzymes involved in the metabolization of R7000 could be more active in the caecum than in the small intestine, illustrating the differences observed between these two organs.

Analysis of the R7000-induced mutation spectrum in the caecum versus time showed that large deletions ($\geqslant$ 3 base

pairs), single (G:C) deletions as well as GC->CG transversions were the first mutatons induced after the end of the treatment. Fifteen days later, the characteristic R7000 mutation specificity, already reported in *E. coli* [42] and small intestine [31], was recovered. Some discrepancies appeared between caecum and small intestine. R7000 induced additions of one base pair in the small intestine but not in the caecum. Inversely, the proportion of GC->CG transversions was 4 times higher in the caecum than in the small intestine. It is interesting to note that in mammalian cells, abasic sites lead to AT->CG, GC->CG and AT->GC events. Since 80% of guanines are mutated, the occurrence of GC->CG transversions suggested that, as previously mentioned for *E. coli* [42], in mouse, abasic sites could be an intermediate step in the repair pathway of R7000 premutagenic lesions.

The mutagenic effects of R7000 were also analyzed after oral administration in Big Blue transgenic mice. In addition to the *lacI* gene, the *cII* gene of the lambda shuttle vector can also be used as a target for mutagenesis in the Big Blue transgenic mouse assay [14]. The advantage of the *cII* gene is to allow a positive selection of the mutants. Similar results are obtained when either *lacI* or *cII* genes are used as target [9]. The frequency of *cII* mutants was significantly 5-fold higher in the stomach of R7000-treated animals than of untreated animals. A significant slight increase (1.7-fold) in the mutant frequency was also observed in the bladder of R7000-treated animals. Thus, R7000 could be considered as a locally acting compound since it induced a more pronounced mutagenic effect at the site of administration. Accordingly, when given orally to rats, it led to squamous cell carcinoma development in the mouth and stomach [36]. However, its carcinogenic effect was evaluated as weak as compared to some nitrosamines. The mutagenic effect observed in the stomach could result from an active metabolization of R7000 by the gastric cells. The product could lead to genotoxic derivatives through the action of acid secretion in the stomach. Likewise, the presence of food and bacteria in the gastric lumen could play a role in the metabolic activation of the compound.

10. Mutagenic properties of nitrofurantoin and nifuroxazide in *lacI* transgenic mice

Nitrofuratoin and nifuroxazide are nitrofuran derivatives widely used in human medicine, nitrofurantoin as first-line prophylaxis therapy for acute or recurrent urinary tract infections and nocosomial urinary tract infections, and nifuroxazide as therapy for acute diarrhoea of bacterial origin [5]. Like other nitrofuran derivatives the two compounds are mutagenic in bacteria [24]. Surprisingly, only a few studies on the in vivo as well as in vitro mutagenic action of these two compounds in higher organisms are available. In vitro, an increase in the frequency of sister-chromatid exchange in Chinese hamster ovary cells exposed to nitrofurantoin has been observed [39], whereas nifuroxazide did not induce mutations at the HGPRT locus in V79 Chinese hamster lung cells using a rat-hepatocyte-mediated metabolic activation system [7]. In vivo, nitrofurantoin was not found to induce micronuclei in reticulocytes from bone marrow of rats [10] and did not show a significant effect in the mouse spot test [8]. However, nitrofurantoin was classified as a borderline case to induce sex-linked recessive lethal mutations in *Drosophila melanogaster* [17]. In addition, the effect of nitrofurantoin on the maturation of spermatozoa in the H test in the mouse was tentatively interpreted by the authors as indicative of a mutagenic effect [11]. As far as nifuroxazide is concerned no data on in vivo genotoxic assays in higher organisms have been published.

The in vivo mutagenic action of nitrofurantoin and nifuroxazide was investigated using the Big Blue transgenic mouse mutation detection system that allows direct determination of in vivo somatic mutation frequency in any tissue or cell type. The *cII* gene of the lambda shuttle vector was used as a target for mutagenesis [28]. We found that oral administration of nitrofurantoin induced a weak but significant mutagenic effect in the *cII* gene from the kidneys of Big Blue transgenic mice. No significant increase in mutants could be evidenced in the other organs tested after oral administration of either nitrofurantoin or nifuroxazide. The doses used per unit weight were comparable to the doses used in humans.

11. Conclusions

The main and possibly only target for activated derivatives of R7000 on DNA is guanine. At least 9 different adducts were detected by the ^{32}P postlabelling technique, illustrating the large spectrum of modifications made.

The extreme mutagenic activity of R7000 in bacteria can be accounted for by at least two factors: A single lesion is sufficient to appreciably induce the SOS system and can thus result in targeted and untargeted mutagenesis (other error-prone repair systems also seem to be involved; see above); in the *lacI* sequence tested, all guanine residues appeared equally effective for R7000 derivatives, indicating that the number of damaged guanines was potentially very high. Other factors such as the efficiency of activation by bacterial nitroreductases are also likely to play a role.

In a mammalian cell test, the CHO/HPGRT assay, R7000 also ranked as one of the most active mutagens tested. This indicates that the endogenous metabolization of R7000 in these cells also leads to adducts efficient at triggering error-prone repair.

In Big Blue mice, when R7000 was administered i.p., the nature of the mutations induced in the *lacI* transgene in the caecum and small intestine was rather similar to that induced in the bacterial *lacI* gene, suggesting that analogous events were taking place for activation, DNA damage and error-prone repair. In particular, R7000 precipitates were observed close to the caecum and the small intestine, suggesting that

the localization of the compound in vivo was critical for determining the target organs.

In the same system, when R7000 was administered per os for five consecutive days at doses of 62.5 mg/kg, an increase in mutation frequency of a factor of 4 was observed in the stomach, but no increase was detected in the other organs tested (unpublished results). This suggests that the biodistribution of R7000 in this case also determined the target organ. At this stage it is interesting to recall that nitrofurantoin given by the same route yielded an increase in mutation frequency of a factor of 2 in the kidneys, where it was shown to be selectively concentrated [38] and not in the other organs tested. In contrast, nifuroxazide, which is found essentially in the serum [19], did not induce an increase in mutation frequency in any of the organs tested.

It thus appears that the biodistribution and selective concentration of nitrofurans is a critical factor in determining increases in mutation frequencies in vivo in the mouse. This observation suggests that caution should be taken when humans are treated with nitrofurans in conditions in which the compounds are specifically concentrated in certain organs, such as the kidneys for nitrofurantoin.

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