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Molecular Basis of 4-Nitroquinoline 1-Oxide Carcinogenesis

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INTRODUCTION

The carcinogenic property of 4-nitroquinoline 1-oxide (4NQO) was first reported by Nakahara *et al.*[1] Indeed, 4NQO induces cancer in various tissues, particularly in the lung,[2] pancreas[3] and stomach.[4] After the publication of Nakahara *et al.*,[1] numerous efforts were devoted by Japanese teams to the study of the biological and molecular properties of 4NQO, and the results can be found in several reviews.[5-7] In particular, studies of numerous derivatives were carried out: the non-carcinogenic character of quinoline 1-oxide, 4-nitroquinoline and 3-methyl-4NQO (3Me-4NQO) demonstrates that the NO_2 group at the 4 position, the N-oxide group at the 1 position and carbon 3 without any substituent are necessary for the carcinogenicity of 4NQO.[6] 4NQO is a precarcinogen, i.e. it has to be metabolized by the cellular machinery to an active ultimate carcinogen which can covalently bind to DNA,[8] particularly to guanine and adenine.[9]

The mechanism of mutagenesis by 4NQO has been studied in prokaryotic[10, 11] and eukaryotic systems[12] and 4NQO has been shown to be specific for base-pair substitutions (90%) principally G to A transitions but also a few G to T conversions and rare substitutions of adenines. The mutagenesis of 4NQO has also been studied with respect to the activation of proto-oncogenes, known to be a target of mutagens and, in some cases, activated by point mutation.[13] Indeed recent studies, using the multistage skin tumor model, with 4NQO initiation and TPA promotion, revealed a mutation of the 2nd guanine (G-A) in the twelfth codon of the c-Ha-*ras* proto-oncogene in some of the 4NQO-induced tumors in mice.[14]

Abbreviations: 4NQO, 4-nitroquinoline 1-oxide; 4HAQO, 4-hydroxyaminoquinoline 1-oxide; 4AQO, 4-aminoquinoline 1-oxide; Ac-4HAQO, 4-acetoxyaminoquinoline 1-oxide; diAc-4HAQO, O,O′-diacetyl 4-hydroxyaminoquinoline 1-oxide; HPLC, high-performance liquid chromatograpy; dGuo-C8-AQO, N-(deoxyguanosin-8-yl)-4-aminoquinoline 1-oxide; dGuo-N2-AQO, 3-(deoxyguanosin-N^2-yl)-4-aminoquinoline 1-oxide; dAdo-N6-AQO, 3-(deoxyadenosin-N^6-yl)-4-aminoquinoline 1-oxide; 3Me-4NQO, 3-methyl-4-nitroquinoline 1-oxide; SSBs, single strand breaks.

Much information has been accumulated concerning the carcinogenic properties of various chemical agents, but many details still remain obscure. Few models exist which permit the identification of the different adducts of a carcinogen and at the same time allow an analysis of the biological consequences of the formation of each adduct. Therefore, it would be of great interest to develop a system which reproduces *in vitro*, the *in vivo* state and allows the formation of adducts on DNA to be controlled, and to use such a system to gain a better understanding of the reaction mechanisms involved in the formation of adducts. Over the past few years, we have developed just such a convenient system for 4NQO by utilizing the monoacetyl derivative of 4-hydroxyaminoquinoline 1-oxide (4HAQO). The system reviewed in this paper permits the analysis of the formation of different 4NQO-lesions at the molecular level and is available for numerous studies on such mutagenesis.

1. Acetyl-4HAQO as a Good Model of the Ultimate Carcinogen

Metabolic conversion of 4NQO into 4HAQO and 4-aminoquinoline 1-oxide (4AQO) is observed in prokaryotic and eukaryotic cells,[15] and of these two metabolites, only 4HAQO is a carcinogen.[6, 16] The enzyme which catalyzes this reaction has been found principally in the cytosol fraction of various organs[17] and has been identified as the DT diaphorase, a NAD(P)H-quinolineoxidoreductase.[18] Since 4HAQO is more carcinogenic than 4NQO,[19, 20] it has been considered as a proximate carcinogen of 4NQO. In 1974, Tada and Tada[21] characterized an enzyme responsible for the *in vivo* activation of 4HAQO as a seryl-tRNA synthetase and postulated seryl-4HAQO as the 4NQO-ultimate carcinogen. With an *in vivo* approach, Enomoto *et al.* demonstrated that the diacetylated derivative of 4HAQO (diAc-4HAQO) could bind covalently and non-enzymatically with DNA, at neutral pH.[22] By means of comparative analysis of the fluorescence properties, we have shown that the adducts formed *in vivo* with 4HAQO and those obtained *in vitro* with the diAc-4HAQO had identical optical properties and we proposed that diAc-4HAQO could be a model of an ultimate carcinogen.[23]

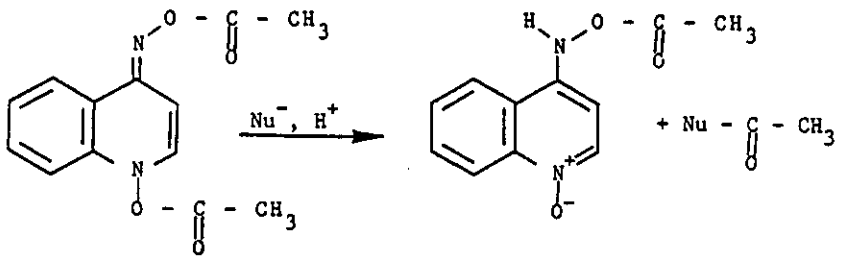

Fig. 1. 4-Acetoxyaminoquinoline 1-oxide formation by acetyl transfer from the O,O'-diacetyl derivative of 4HAQO to a nucleophile.

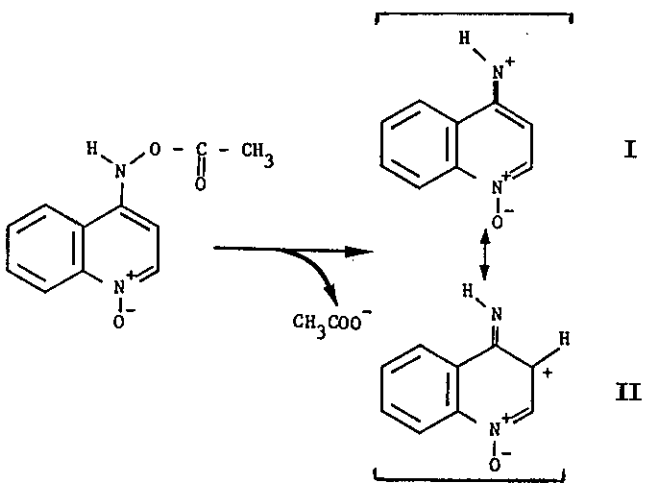

Fig. 2. Nitrenium (I) and carbocation (II) formation from 4-acetoxyaminoquinoline 1-oxide.

Since then, however, the mechanism of action of this compound has been shown to consist of two steps, and we believe that the first reaction has no *in vivo* relevance. First, it transfers an acetyl group to nucleophiles such as the sulfhydryl group of dithiothreitol to yield 4-acetoxy-aminoquinoline 1-oxide (Ac-4HAQO)(Fig. 1), which is a very reactive electrophilic compound. Secondly, this is then able to bind to nucleosides by losing the O-acetyl group (Fig. 2).[24] This derivative is also structurally similar to the postulated seryl-4HAQO and it has been suggested by Kawazoe *et al.*[25,29] that this monoacetyl derivative of 4HAQO could be a good model.

Initially, we showed that Ac-4HAQO was 2- to 3-fold more reactive with DNA than diAc-4HAQO,[26] and then we demonstrated that the adducts formed *in vivo* with 4HAQO and *in vitro* with Ac-4HAQO had the same chromatographic properties.[27] These results lead us to accept Ac-4HAQO as a relevant *in vitro* substitute for the ultimate metabolite of 4NQO. Furthermore, *in vitro* DNA-modification is technically easy and controllable; diAc-4HAQO prepared by the method of Kawazoe[27,28] is incubated with DTT in DMSO as solvent. The Ac-4HAQO formed after a 15 min incubation at room temperature is added to the DNA solution.

2. *Identification of Adducts*

According to the Japanese chromatographic investigations, 4NQO induces principally 3 stable adducts; 2 guanine adducts (QGI and QGII) and one adenine adduct (QAII).[9] They also found a quinoline derivative, 4AQO, in the acid hydrolysis and suggested that this had been formed from an acid-unstable guanine adduct. With this approach the structure of one adduct (QAII) has been proposed as being 3-(adenin-N1 or N6-yl)-4AQO.[30] We used Ac-4HAQO as an ultimate reactant to investigate the chemical structure of the 4NQO adducts.

Three adducts have been identified in Ac-4HAQO-modified-DNA and have been shown to be present in DNA modified *in vivo* with 4HAQO or 4NQO.[27] Indeed the high-performance liquid chromatographic (HPLC) properties and the spectrophotometric characteristics of the adducts obtained *in vivo* are identical to those of the identified *in vitro* adducts.

First, we identified the major adduct obtained by the reaction between Ac-4HAQO and deoxyguanosine (Fig. 3) as the N-(deoxyguanosin-8-yl)-4AQO adduct (dGuo-C8-AQO).[24] A few years later, Tada *et al.* showed that the compound QGI was identical to this C8 adduct.[31] After enzymatic hydrolysis of a large amount of Ac-4HAQO-modified DNA and semi-preparative HPLC, we were able to purify and identify two other adducts; 3-(deoxyguanosin-N^2-yl)-4AQO (dGuo-N2-AQO) and 3-(deoxyadenosin-N^6-yl)-4AQO (dAdo-N6-AQO) (Fig. 3).[32] The two adducts were not formed efficiently with free nucleosides,[24] but were with double-stranded DNA.[32]

With these data it is possible to propose a reaction mechanism for the formation of 4NQO adducts. Firstly,

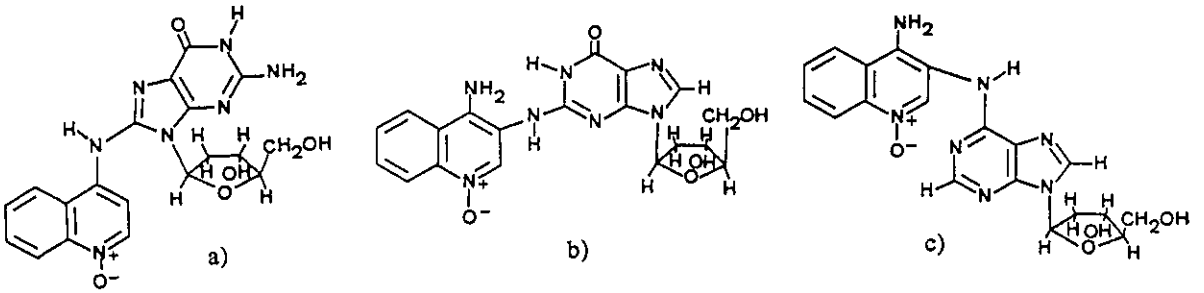

Fig. 3. Structures of 4NQO adducts. a) dGuo-C8-AQO; b) dGuo-N2-AQO; c) dAdo-N6-AQO.

it is tempting to draw an analogy between Ac-4HAQO and the ultimate carcinogens of the arylamide series. Indeed, in the well known case of N-acetoxyacetylaminofluorene, the covalent binding of the fluorene ring occurs through displacement of the O-ester function.[33] In the same manner, we assume that Ac-4HAQO, through loss of the acetate group, gives rise to an arylnitrenium or a carbocation and both reactive forms will react with DNA (Fig. 2). These two forms, i.e. the 4-quinolenium and the 3-quinoline-carbocation, can account for the formation of 4NQO adducts, essentially on purines.[24]

Effectively, the dGuo-C8-AQO adduct results from the attack of the nitrenium ion on the C8 position of guanine, whereas the dGuo-N2-AQO and the dAdo-N6-AQO adducts result from the carbocation attack on the guanine N2 and the adenine N6 positions, respectively.[32] Thus, the 4-nitro group which gives rise to the nitrenium is crucial for adduct formation, as is the N-oxide group. Indeed the Ac-4HAQ compound, without the N-oxide, is unable to react with DNA.[34] The electron-donating effect of the N-oxide is responsible for the labilization of the N-acetoxy bond. In a similar way, the carbon 3 of the quinoline nucleus plays an important role in N2 and N6 adduct formation.

Supporting evidence for this scheme is provided by the weak carcinogenicity and mutagenicity of 3Me-4NQO.[35] The bulky methyl group probably reduces the reactivity of the carbocation leading to formation of the N2 and N6 adducts. No direct evidence of lack of formation of these adducts has been obtained but a comparison of the induction of single strand breaks (SSBs) by 4NQO and 3Me-4NQO shows that one particular type of SSBs is completely absent in the case of the methyl derivative.[36-38]

One point about the adduct is still unclear, i.e., whether it is the acid-unstable adduct that gives rise to 4AQO formation described by Tada and Tada.[9] By utilizing enzymatic hydrolysis, 4AQO was also found in DNA modified *in vitro* with Ac-4HAQO and *in vivo* with 4NQO. The formation of 8-hydroxyguanine residues has also been observed after DNA modification with 4HAQO in the presence of seryl-AMP,[39] and the authors suggested that active oxygen species or radicals might not take part directly in the formation of this lesion. To explain this observation, they postulated the existence of an unstable adduct on the guanyl-N7,[40] but this has never been demonstrated. Nevertheless, this postulated adduct could explain the formation of 4AQO in hydrolysis of the modified DNA.

On the other hand, dGuo-C8-AQO was shown to be unstable and to decompose during enzymatic hydrolysis and especially during alkaline phosphatase action at pH 8.[27] Instability of the imidazole ring in an alkaline medium has already been described for certain adducts on the guanyl-C8[41-43] and also on the N7 of the same base.[44] Two types of breakage have been described in the literature for different C8 adducts, i.e. through the 7,8 or through the 8,9 guanine bond.[42,43] We carried out a study to identify the degradation product of dGuo-C8-AQO and demonstrated that hydrolysis occurred at the 7,8 guanine bond to open the imidazole ring.[45] There is no direct evidence for the *in vivo* existence of this adduct. Although it is observed in the enzymatic hydrolysis of *in vivo* 4NQO modified DNA, this product may in fact be an artifact created during the analysis.

3. Quantitative Aspects of Adduct Formation

To get a better understanding of 4NQO action, several quantitative approaches have been developed with Ac-4HAQO modified DNA. Firstly the total amount of bound quinoline derivatives has been evaluated using tritium-labeled quinoline.[26] This method gives us the molar extinction coefficient at 365 nm of bound carcinogen in order to allow a direct spectrophotometric titration. Moreover, dGuo-C8-AQO exhibits a specific shift under alkaline conditions. The absorption band is located

at 365 nm under neutral conditions, while at basic pH, the absorption maximum appears at 440 nm. This spectrophotometric property allowed us to develop a rapid method for evaluation of the amount of this adduct on DNA.[46] For evaluation of N-2 guanine and N-6 adenine adducts, the 4NQO modified-DNA is first enzymatically digested to obtain nucleosides and then the 4NQO adducts are quantified by HPLC. Both methods are complementary and give similar data.[26]

By utilizing the method described above, we were able to show that the reaction of Ac-4HAQO is 2 to 3 times more efficient with denatured DNA than with native DNA. Moreover the analysis of adduct formation shows that the C-8 adduct represents about 70% of the total modification of denatured DNA while it is only present at 25% in native DNA. Therefore, the secondary structure of DNA influences the formation of 4NQO adducts quantitatively and qualitatively. Similar observations had already been described with other carcinogens.[47]

In native DNA the complete pattern of adduct modification has been obtained; dGuo-N2-AQO is the predominant lesion (50%), and Ado-N6-AQO is a minor adduct (about 10%). A similar pattern is observed with DNA modified *in vivo* by 4NQO or 4HAQO. This data supported the choice of this system using Ac-4HAQO as an *in vitro* ultimate carcinogen model. Moreover, the 4AQO is observed in both *in vivo* and *in vitro* analysis.

4. Relevance to Previously Used in vivo System

When DNA modified with 4NQO *in vivo* is analyzed by sedimentation velocity measurement under alkaline conditions, SSBs are observed.[48] On the basis of this finding, 4NQO had been proposed to cause SSBs *in vivo* by inducing free radicals[49]; oxygen radicals formed by X-rays are known to induce similar damage. However, a more plausible explanation of these observed SSBs is now possible.

This explanation arises from Walker's observation concerning the influence of 4NQO on cellular NAD levels.[50] SSBs are known to stimulate *in vivo* poly(ADP ribose)transferase activity and consequently to deplete cellular NAD. With 4NQO, no fall in NAD level is observed, as is the case with UV-induced damage. So it would appear that no SSBs are present *in vivo*, and according to Walker, any SSBs formed during excision-repair must be protected by protein as in the case of UV lesions.

From results obtained by the same technique of alkaline sucrose gradient analysis, Walker suggested that SSBs arise from alkali-labile lesions in 4NQO-treated human cells, principally because he was able to identify two types of SSBs. Class I SSBs form after a brief alkaline treatment and are rapidly removed, both in normal and XP fibroblasts, independently of α-polymerase.[48] Class II SSBs are alkali-stable under short-term lysis conditions, occur with a frequency of four times that of class I lesions, and are repaired in a slower and α-polymerase-dependent manner.[36-38] Walker found that both classes of SSB are removed by the repair machinery in hours rather than minutes, like SSBs induced by X-rays, and this speed of repair is more reminiscent of pyrimidine dimers or alkylation damage than *in vivo* SSBs.[51]

Therefore, it would seem that the majority, if not all, of the observed SSBs occur in the alkaline gradient and do not represent direct *in vivo* 4NQO-induced SSBs, but are created rather by 4NQO-metabolism through several bulky adducts. It would be of interest to know which adducts give rise to class I and class II SSBs. Some data, which will be discussed later on, indicate that 4NQO-SSBs obtained after brief lysis may result from apurinic sites formed from an unstable adduct[52] which under alkaline conditions leads to chain breaks.

Indeed, class I lesions are repaired in XP cells and one would expect repair of apurinic sites in these cells, except perhaps those from complementation group D.[53] Furthermore, in collaboration with Abbondandolo *et al.* (in preparation), we found *in vitro* Ac-4HAQO-induced SSBs and demonstrated that they were essentially experimental artefacts, e.g. apurinic site cleavage caused by the electric field, the electrophoresis buffer and the mechanical stress caused by the migration of DNA through the agarose matrix. We evaluated the rate of depurination, via an unstable adduct, as 1 apurinic site per fifty adducts. It appears that the SSB class I actually induced by 4NQO treatment *in vivo* could be essentially due to apurinic sites, known to be formed quite weakly under neutral conditions.[54] So it is clear that all DNA lesions, including class I SSBs, induced by 4NQO occur through metabolic activation and adduct formation. Thus, the model of Ac-4HQO seems perfectly adequate to account for the creation *in vitro* of each of these lesions.

In view of the knowledge of 4NQO adducts and mechanisms of reaction, it is tempting to classify the different adducts into the two classes of alkali-labile lesions mentioned above. Indeed, with 3Me-4NQO a total absence of class II SSBs is observed and thus only the very alkali-labile lesions (class I) are seen.[37, 38] This derivative, by virtue of the methyl group, may form adducts only through the nitrenium species. Consequently it would seem that the class II lesions arise via the carbocation species. So the dGuo-N2-4AQO and dAdo-N6-4AQO adducts could be responsible for the class II SSBs, being partially resistant to alkali and repaired by the excision repair system. In addition, the dGuo-C8-AQO adduct represents about 20–25% of total DNA modification *in vivo*. This value is in good agreement with the fact that

the class I lesion accounts for only a fifth of the total alkaline lesions.

However, the postulated guanine C8 adduct of 3Me-4NQO visualized as SSBs class I appears not to be crucial for mutagenesis and carcinogenesis. Indeed the methyl derivative of 4NQO is a weak carcinogen and mutagen.[55] This is in agreement with the earlier discussion which postulates that these lesions induce apurinic sites and are weakly mutagenic. It is very tempting to conclude that the 4NQO C8 adduct has the same properties.

5. *Work in Progress*

A very convenient *in vitro* system is now available which allows the easy modification of DNA, the control of the rate of modification and the determination of the ratio of the different adducts. Recently we observed that the reaction of Ac-4HAQO with native DNA, when carried out at low temperature (0 to $-10°C$) and low ionic concentration, occurs mainly (about 80%) at the N2 position of guanine (Galiègue-Zouitina, unpublished data). By using these reaction conditions, we studied the formation of dGuo-N2-AQO in a prokaryotic system perfected by Fuchs *et al.*[56] The data show that this lesion exhibits highly mutagenic properties and that the mutations induced are mainly base substitutions only on guanine residues (Daubersies, in preparation). Some studies are in progress to analyze the dGuo-C8-AQO route of mutagenesis through the reaction of Ac-4HAQO with denatured DNA.

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