Carboxymethylating/methylating agents associated with dietary nitrosating agents: potential role in gastrointestinal carcinogenesis

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From the point of view of a chemical toxicologist one of the most intriguing aspects of nitrosation reactions is their ability to transform innocuous molecules into agents that display a wide range of biological activity. The archetypical example is the conversion of dimethylamine (DMA) into N-nitrosodimethylamine (NDMA): DMA displays little biological activity but NDMA is a potent liver carcinogens. Over the past fifty years the mechanism by which this occurs has been intensively studied and involves the generation of a highly reactive methylating agent that modifies DNA giving rise to adducts which, if left unrepaired, lead to miscoding sequences that can lead to functional mutations. There are many variations on the theme of N-nitroso compounds (nitrosamines, nitrosamides, nitrosoureas, nitrosoguanidines, and so on) but a common thread running through this area of research is that nitrosation leads to the formation of an N-nitroso derivative which can either decompose to give a DNA-damaging (alkylating) agent, or, do so after some sort of metabolic activation.

This view of nitrosation has given rise to several notions: 1. N-nitroso compounds can be formed in foods or other consumer products and may pose a cancer risk to humans, and, 2. Eliminating or minimising exposure to N-nitroso compounds would lead to a reduction in cancer risk. Some cancer risks have been associated with exposure to N-nitroso compounds – as, for example, with industrial reagents and solvents and tobacco use. However in other areas of human cancer risk such links have proved elusive. Most notably, the role of N-nitroso compounds in gastrointestinal cancer has proved very difficult to resolve. There are many experimental models in which N-nitroso compounds (such as MNNG and the N-nitroso bile acid conjugates) can be used to induce cancers of the GI tract but there is little evidence that exposure to such materials is directly involved in the induction of human cancers. However, experimental models have given us a number of clues which allow a more subtle view of the involvement of N-nitrosation in human GI tract cancers.

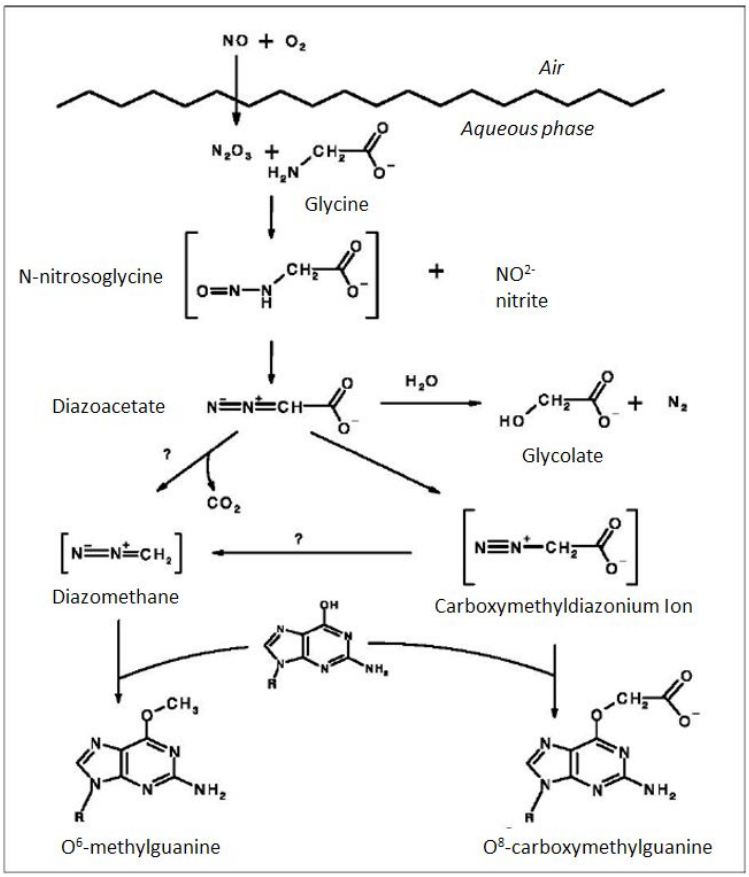
N–Nitrosoglycocholic acid (NOGC) was first synthesised in 1979 as part of a programme in Steve Tannenbaum's group at MIT[1]. NOGC was found to not only induce gastric cancers in experimental animals but also the precancerous lesions typical of the human disease[2]. Early studies on DNA adducts formed by NOGC led to the identification of N-7-carboxymethylguanine, which had also been found by Longnecker and colleagues in DNA treated with azaserine[3]. This result suggested that the carcinogenic properties of NOGC were driven predominantly by the fact that it was an N-nitroso

glycine derivative rather than a bile acid. Azaserine is actually an N-nitrosoglycine derivative in disguise. Initial studies focussed on the formation of carboxymethyl adducts as these were thought to be characteristic enough to be the basis of biomonitoring methodology. However, it became apparent that DNA exposed to a range of nitrosated glycine derivatives also contained methyl adducts. The ability to form both carboxymethyl- and methyl-DNA adducts turns out to be general property of nitrosated glycines. The nitrosated glycines comprise a class of compounds that include N-nitrosamines (e.g. N-nitrososarcosine), N-nitrosamides (e.g. NOGC, N-nitrosopeptides) and diazoacetates (e.g O-diazoacetyl-L-serine [azaserine]). In fact the simplest form of nitrosated glycine is diazoacetate (usually in its potassium salt known as KDA) first reported in 1908[4] but little studied in the ensuing century.

The formation of methyl DNA adducts by nitrosated glycines is intriguing as it suggests that promutagenic adducts such as O6-methyl-2'-deoxyguanosine (O6MedG) can come from sources that are not obvious methylating agents. This might explain why it has proved so difficult to consistently relate levels of O6MedG in human tissues to particular dietary or environmental risk factors. However, the major DNA modifications formed by nitrosated glycines are the carboxymethyl adducts and these have formed the basis of biomonitoring assays. Thus antibody-based assays including immunoaffinity-HPLC[5] and immunoslotblot assays[6] and immunohistochemistry[7] have all been developed and used to quantitate the characteristic adduct, O6-carboxymethyl-2'-deoxyguanosine (O6CMdG) in various studies. More recently, sensitive mass spectrometry-based assays have become available and these too have confirmed the presence of O6CMdG in human tissues[8].

An early observation was that O6CMdG appeared to be resistant to the various O6-methylgaunine methyl transferases that exist in bacterial and mammalian cells to remove this toxic methyl lesion[2].

How might carboxymethyl adducts be formed in vivo? The following scheme shows how glycine can be transformed into both methylating and carboxymethylating agents. The key point here is that the obligatory imtermediates – N-nitrosoglycine, diazoacetate, carboxymethyl-diazonium ion and diazomethane are all very short-lived species at physiological pH. Thus the two O6-alkyl adducts (along with the corresponding N7-alkylguanine adducts) are the 'smoking gun' which shows that the nitrosation reaction had occurred.



The large body of work on endogenous nitrosation carried out by Bartsch, Ohshima and colleagues[9] demonstrated that the human body had plenty of potential to carry out the type of nitrosation described in the scheme. In those studies the detectable surrogate for endogenous nitrosation was L-proline leading to formation of N-nitroso-L-proline which is stable and non-carcinogenic being quantitatively excreted in urine.

What is the evidence that carboxymethylation and methylation from glycine is likely to be of significance for human health? N-nitroso glycine derivatives are consistently mutagenic and carcinogenic is both *in vitro* and *in vivo* assays[10]. The mutation spectrum induced by KDA in a functional p53 mutational assay is quite distinct from that induced by MNU suggesting that carboxymethylation is contributing substantially to the biological activity[11]. Moreover, the spectrum of mutations in p53 exposed to KDA in vitro is very similar to the spectrum of p53 mutations observed in human GI cancers. Site-specific incorporation of O6CMdG into plasmids resulted in mispairing upon replication with a propensity for GC-AT transitions and GC-TA transversions[12]. Whether this activity is driven by the bulk of the O6-carboxymethyl group or its charge, or a combination of the two, is not yet known. Other carboxymethyl adducts formed by KDA have been identified and display distinctive mutagenic activity[13].

Studies in human volunteers consuming controlled amounts of red meat showed that levels of O6CMdG were raised in exfoliated colonic cells of those subjects who consumed the highest levels of red meat. The simplest explanation is that increased consumption of meat protein resulted in increased levels of glycine leading to increased levels of DNA-carboxymethylation. Whether this is predictive of the increased risk of colorectal cancer awaits the outcome of the large prospective studies such as EPIC where stored DNA could be analysed for carboxymethyl adducts. Here the technology has let us down in that efforts to produce a monoclonal antibody for O6CMdG have failed at several attempts. There was no problem in making a polyclonal serum5 so this seems surprising.

It might seem curious that the most common and simplest amino acid glycine should be capable of being converted in a potent mutagen and carcinogen by a simple nitrosation reaction. Can it really be the case that this pathway contributes substantially to the risk of developing gastrointestinal cancer? If so, why hasn't this been seen a long time ago? It is probably the fact that this pathway is so prevalent that explains why it hasn't come to the fore. Background levels of O6MedG have been seen in human DNA for many years and exposure to known methylating agents such as NDMA did not seem to explain this. If the source of O6MedG is nitrosated glycine then this would not have been at all obvious - least of all to myself, who did not spot it until several years of research in the area. If nitrosated glycine is contributing to the burden of mutagenic DNA damage can we do anything about it? The risk of cancer from this pathway is affected not only by the unavoidable consumption of glycine but also by the extent of endogenous nitrosation , which is itself influenced by consumption of nitrate as well as endogenous synthesis of reactive nitrogen species during inflammation. If one adds into this equation the role of DNA repair (or lack of it) as well as other factors then it is perhaps not surprising that it would be difficult to identify a dietary question or parameter that could measure this pathway. This said, is something like O6CMdG well enough characterised to merit further investigation as a biomarker? Are the available methods sensitive and robust enough to be used in a prospective molecular epidemiological study using EPIC samples (or other stored DNA)? Looking further ahead, could such a marker (or set of related markers) be useful in intervention studies (reducing meat/nitrate intake or raising vegetable intake)? These are the questions to address in the course of our discussions.

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[1] Shuker DEG, Tannenbaum SR, Wishnok JS. N-nitrosobile acid conjugates 1. Synthesis, chemical reactivity and mutagenic activity. *J Org Chem* 1981;46:2092-6

[2] Shuker DEG, Margison GP. Nitrosated glycine derivatives as a potential source of O6- methylguanine in DNA. *Cancer Res* 1997;57:366-9

[3] Zurlo J, Curphey TJ, Hiley R, Longnecker DS. Identification of 7-carboxymethylguanine in DNA from pancreatic acinar cells exposed to azaserine. *Cancer Res*. 1982, 42:1286-8.

[4] Müller E. Uber pseudo diazoessigsaüre (On pseudo-diazoacetic acid). *Chem Ber* 1908;41:3116-39

[5] Harrison KL, Fairhurst N, Challis BC, Shuker DEG. Synthesis,characterization, and immunochemical detection of O6-(carboxymethyl)-2'-deoxyguanosine: a DNA adduct formed by nitrosated glycine derivatives. *Chem Res Toxicol* 1997;10:652-9

[6] Cupid B, Zeng Z, Singh R, Shuker DEG. Detection of O6-carboxymethyl-2'-deoxyguanosine in DNA following reaction of nitric oxide with glycine and in human blood DNA using a quantitative immunoslot blot assay. *Chem Res Toxicol* 2004;17:294-300

[7] Lewin,M.H., Bailey,N., Bandaletova,T., Bowman,R., Cross,A.J., Pollock,J., Shuker,D.E.G. and Bingham,S.A. Red meat enhances the colonic formation of the DNA adduct O6-carboxymethyl guanine: implications for colorectal cancer risk. *Cancer Res*., 2006, 66, 1859–1865

[8] Vanden Bussche J, Moore SA, Pasmans F, Kuhnle GG, Vanhaecke L. An approach based on ultra-high pressure liquid chromatography-tandem mass spectrometry to quantify O^6-methyl and O^6-carboxymethylguanine DNA adducts in intestinal cell lines. *J Chromatogr A*. 2012, 1257:25-33

[9] Ohshima H, Bartsch H. Quantitative estimation of endogenous nitrosation in humans by monitoring N-nitrosoproline excreted in the urine. *Cancer Res*. 1981;41:3658-62.

[10] Puju, S., Shuker, D. E. G., Bishop, W. W., Falchuk, K. R., Tannenbaum, S. R., and Thilly, W. G. Mutagenicity of N-nitroso bile acid conjugates in *Salmonella typhimurium* and diploid human lymphoblasts. *Cancer Res*., 1982,42: 2601-2604: Busby, W. F., Jr., Shuker,D. E. G., Charnley, 0., Newberne, P. M., Tannenbaum,
S. R., and Wogan,G. N. Carcinogenicityof the nitrosatedbile acid conjugates, N-nitroso-glycocholic acid and N-nitrosotaurocholic acid. *Cancer Res.,* 1985,45:1367-1371,

[11] Gottschalg E, Scott GB, Burns PA, Shuker DE. Potassium diazoacetate-induced p53 mutations in vitro in relation to formation of O6-carboxymethyl- and O6-methyl-2'-deoxyguanosine DNA adducts: relevance for gastrointestinal cancer. *Carcinogenesis*. 2007;28:356-62

[12] Ponnada S, Burns PA,, Xu Y-Z, Shuker DEG. O6-carboxymethylguanine: A potent mutagenic adduct characterised using an Amplification Refractory Mutation System –Polymerase Chain Reaction assay following site-specific incorporation into a p53 cDNA sequence. Unpublished manuscript

[13] Swanson, AL Wang, JS Wang, YS. In Vitro Replication Studies of Carboxymethylated DNA Lesions with *Saccharomyces cerevisiae* Polymerase eta. *Biochemistry*. 2011, 50, 7666-7673