Bone marrow genotoxicity of N-methyl, N-nitrosourea (NMU): n-alkanols as sister chromatid exchange (SCE) anti-inducers

K.-W. Stahl[1] and U. Bayer[2]

Institut de Cancérologie et d'Immunogénétique, Laboratoire d'Immunopharmacologie Moléculaire, 16 Av. P.-V.-Couturier, F-94800 Villejuif (France), and Institut National de Recherches sur le Cancer, CNRS, BP No. 8, F-94800 Villejuif (France), November 9, 1982

Summary. The in vivo SCE test was used to demonstrate significant inhibition of NMU bone marrow genotoxicity by pretreatment of Chinese hamsters with n-alkanols. Our findings exclude a loss of intracellular DNA alkylation potential through a competitive direct reaction of NMU with the weakly nucleophilic polar end of the n-alkanols, but not through methylations of nucleophilic membrane sites possibly liberated by structural modifications which the membrane-active amphiphilics induce.

The practical use of alkylating agents such as N-alkyl, N-nitrosoureas in cancer chemotherapy will depend on the answer to the question, whether their mutagenic/carcinogenic effects and their anti-tumor activity can be further dissociated. One way of investigating possible dissociations may be an additional treatment with anti-genotoxic compounds which have little or no intrinsic activity in the aformentioned respect.

In work described in a previous paper[3] we showed, in vivo and in vitro, that the amphiphilic compound and solubilizer, glycerine-polyethylenglycol-ricinoleate (Cremophor EL®, BASF Ludwigshafen) markedly reduces N-methyl, N-nitrosourea (NMU) induced sister chromatid exchanges (SCE). In this communication we present further, albeit indirect, evidence strongly suggesting that membrane-active[4,5] amphiphilics do not reduce NMU genotoxicity as direct competitive inhibitors of DNA alkylations and methyl-group acceptors on their polar end. In vivo the SCE anti-inducer effect of n-alkanol pretreatments is found to be dose-dependent, and it is associated but not directly correlated with the chain lengths of three homologous alcohols ($C_8 \gg C_6 > C_{10}$).

Materials and methods. According to the method of Allen et al.[6], 5-bromo, 2-deoxyuridine (BrdU) tablets of 50 mg pure material (Serva, research grade) were implanted s.c. into the necks of 12–18-week-old Chinese hamsters *(Cricetulus griseus)* weighing about 30 g, from the IRSC breeding center, Villejuif. A microcrystalline form of NMU, if not otherwise stated, was dissolved in sterile physiological saline (0.9% NaCl) immediately before i.p. application. For i.p. administration n-hexanol, n-octanol and n-decanol (Fluka, puriss. quality) were emulsified in 0.9% NaCl by sonification (30 sec, Branson sonifier, model No. 130, maximum power 400 W, 20 kHz frequency) at concentrations ranging from 3×10^{-1} M to 10^{-2} M. Animals which had or had not been pretreated with n-alkanols 30 min before, received NMU 2 h after the implantation of the BrdU tablet. 24 h after NMU treatment the animals were sacrificed using ether; they received a single dose of colchicine (10 mg per kg b.wt) 2 h before. Bone marrow smear preparations and SCE scores were carried out in the double blind manner according to details and criteria published by Marquardt and Bayer[7]. Unless otherwise stated, 50 second-division bone marrow metaphases were recorded from each animal. Duncan's multiple variate analysis procedure[8] was employed for comparing SCE scores of the NMU treated groups in 1 series of experiments, taking $p < 0.05$ as limit of significance.

Results. In accordance with previous results from in vitro experiments[3] bone marrow genotoxicity of NMU followed a sigmoideal dose response curve with a medium linear range as shown in figure 1. An experiment was designed to investigate whether the genotoxic efficiency of NMU was determined by the highly reactive[9] compound's movement into the cell: NMU was injected i.p. after dissolving the dose of 5×10^{-5} moles per kg b.wt in a volume of distilled

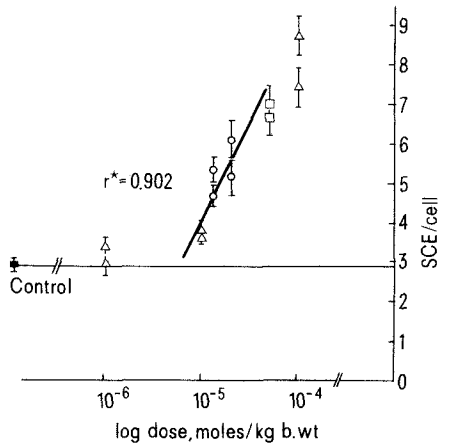

Figure 1. In vivo induction of SCE (ordinate) by NMU (abscissa) recorded on a per cell basis from 50 2nd-division metaphases of 1 donor in 3 series of experiments (△, □, ○). r* = linear correlation coefficient for the range from 10^{-5} to 5×10^{-5} moles NMU per kg b.wt given i.p. The SCE value (■) (2.85 ± 0.07 SEM) was calculated as the mean of 10 pooled records from the total number of untreated animals scored in every experiment described in this report. Bars indicate standard error of the mean (± SEM).

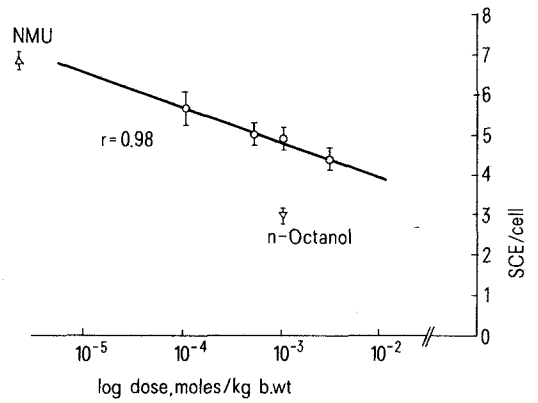

Figure 2. Decrease of NMU (5×10^{-5} moles per kg b.wt) induced SCE rates in bone marrow cells (ordinate) by pretreatment (i.p. injections at t = −30 min) of the animals with 4 different doses of n-octanol (abscissa). n-Octanol (▽) and NMU (△) represent mean SCE values of the separate treatment controls at 10^{-3} and 5×10^{-5} moles per kg b.wt respectively. Each point represents the mean SCE score recorded on a per cell basis from 2×50 2nd-division metaphases pooled from 2 animals. Bars indicate (± SEM).

water corresponding to 5% of the animal's b.wt. This lead to a SCE score (table 1.1) which was markedly higher than those obtained under the standard experimental conditions described in Methods (fig. 1 and table 1.2). However, when animals from the same group had been additionally pretreated with 2 homologous n-alkanols (C_8 and C_{10}), significantly lower SCE rates (table 1.1) were found.

Because of the stronger effect of n-octanol pretreatment this n-alkanol was further examined in a dose-response experiment. Results are given in figure 2. A dose of 5×10^{-5} moles NMU per kg b.wt induced a mean SCE score per cell of 6.7. After n-octanol pretreatment this value decreased linearly with increasing doses of n-alkanol, plotted on a log scale.

It has been shown with liposomes that lipid hydrolysis by various phospholipases is increased in the presence of n-alkanols and that the chain length of the alcohols is an essential factor for this enhancement effect, which increases from C_4 to C_8, and is progressively reduced from C_9 to C_{12}[4]. Such observations prompted the following experiment, the results of which are shown in table 1.2. Three groups of hamsters received 10^{-3} moles per kg b.wt of either n-hexanol or n-octanol or n-decanol followed or not by 5×10^{-5} moles NMU per kg b.wt, using the standard protocol described in Methods. In comparison with untreated controls (fig. 1) no intrinsic effect on the SCEs was noted from n-alkanol treatment. However, all 3 homologous n-alkanols lowered NMU bone marrow genotoxicity. The strongest SCE anti-inducer effect was clearly found for the C_8 chain length. At equimolar doses, n-hexanol and n-decanol were significantly less active, although the protective effect of the latter was not found to be significant.

Discussion. Our studies showed that in vivo neither distilled water nor 3 homologous (C_6, C_8, C_{10}) n-alkanols increased SCE above the score of untreated controls, but they modified the genotoxic response to NMU treatment in the bone marrow of Chinese hamsters.

NMU is a very reactive[9] compound. So, effects on DNA due to alkylation will depend on the number of molecules reaching the cell nucleus without reacting with the numerous nucleophilic sites showing highly preferential[10] reactivity towards N-nitroso compounds; sites, which the molecules will encounter most frequently on their trans-membrane passage as PO_4^{2-}, SO_4^{1-}, COO^- and R-OH groups. This is confirmed by experiments showing that the DNA alkylation rate in human fibroblasts exposed to alkylnitrosoureas is less than 1% of the value obtained after direct reaction of nitrosoureas with purified DNA from the same cells[10]. Therefore, any mechanism facilitating NMU movement into the cell should increase DNA lesions which are recorded from SCE scores, and any mechanism favouring NMU decomposition on the molecules' passage to the nucleus should decrease the number of lesions.

By a mechanism known as solvent drag[11] passive transmembrane transport kinetics can be increased, because NMU movement into the cell is a resultant of the molecules' diffusion and of the solvent movement. 1.5 ml of distilled water injected i.p. together with NMU into Chinese hamsters weighing 30 g can exert a rather potent solvent drag on NMU, thus procuring a straightforward explanation for the observed increase of NMU genotoxicity by hypotonic shock.

On the other hand, n-alkanol pretreatment significantly lowered NMU genotoxicity. As this effect was found to be dose-dependent, one might argue that the weakly polar ends of the amphiphilic alcohols act as carbenium ion capture sites and as direct competitive inhibitors of DNA alkylation. Three major arguments stand against this. Firstly, no ether product could be isolated from incubations[3] of both compounds, NMU and n-octanol, in equimolar amounts at pH 7.2 and 20 °C. Secondly, results from chemical in vitro reactions obviously do not supply conclusive evidence for the in vivo situation. But additional support against the aforementioned hypothesis comes from our findings, namely that the protective effect of the 3 homologous n-alkanols is not correlated with increasing nucleophilicity of the R-OH groups, i.e. decreasing carbon atom chain length. Furthermore, a single experiment with 1 animal (results not shown) showed a narcotic but no SCE anti-inducer effect of a pretreatment with 10^{-3} moles ethanol per kg b.wt. Thirdly, in vitro, spacy amphiphilics such as nonsaturated fatty acid esters in cis-configuration have been found to be more potent SCE anti-inducers than n-octanol[3], although they do not present any nucleophilic groups susceptible to react with NMU.

The exact mechanisms by which n-alkanols and other membrane-active amphiphilics[3] reduce NMU genotoxicity are not clear. Two explanations are conceivable, but our findings offer no direct evidence. Firstly, n-alkanols may lower the trans-membrane bioavailability of NMU by increasing its lipid/water partition coefficient, in analogy with Blank's results[12], which demonstrated that compounds penetrate skin less rapidly from vehicles in which they are more soluble. Secondly, membrane-active amphiphilics have been postulated to induce membrane structure modifications[4,5,13,14] with subsequent steady-state fluorescence polarization changes in ^{1,6}diphenyl-,^{1,3,5}hexatriene labeled platelets[13,14], surface antigen capping in lymphocytes[5], enhancement of enzymatic phospholipid hydrolysis in platelets[13,14] and liposomes[4], and increased agar penetration of *Schistosoma mansoni* cercariae[16]. The optimum n-alkanol chain length appears to be C_8 for both the enzymatic lipolysis increase in liposomes[4] and the protection against NMU induced SCE. From this striking similarity it could be argued that n-alkanols might decrease nuclear bioavailability of NMU through liberation of additional nucleophilic sites after enhanced membrane lipid hydrolysis.

Agents which decrease genotoxicity of N-nitroso compounds by topocellular alkylation shifts could be useful oncopharmacological tools to probe the significance of other-than-nuclear target sites for the anti-tumor activity[16] of methylating N-nitrosoureas.

Protective effect of n-alkanol (10^{-3} moles per kg b.wt) pretreatment on in vivo induction of SCE by NMU (5×10^{-5} moles per kg b.wt i.p.)

Treatment	Scored cells	No. of animals	SCE/cell ± SEM
I			
Control^{H2O}	50	(2)	2.9 ± 0.2
n-Octanol	25	(1)	3 ± 0.1
+ NMU^{H2O}	50	(2)	9.2 ± 0.5 [a]
n-Octanol + NMU^{H2O}	50	(2)	5.3 ± 0.4 [a]
n-Decanol + NMU^{H2O}	50	(2)	8 ± 0.6 [a]
II			
n-Hexanol	25	(1)	2.9 ± 0.3
n-Octanol	25	(1)	3.0 ± 0.1
n-Decanol	25	(1)	2.8 ± 0.3
+ NMU	100	(2)	7 ± 0.3 [a]
n-Hexanol + NMU	100	(2)	6.0 ± 0.3 [a]
n-Octanol + NMU	100	(2)	5.1 ± 0.3 [a]
n-Decanol + NMU	100	(2)	6.4 ± 0.3

Except for the first series of experiments (I), where the animals were exposed to hypotonic shock receiving 5 b.wt % distilled water (control^{H2O}, NMU^{H2O}), the standard treatment protocol described in Methods was used. SCE data are given as means (± SEM) recorded on a per cell basis from 25, 50, or 100 2nd-division metaphases either derived from 1 or pooled from 2 animals. Chinese hamsters submitted to the combined n-alkanol/NMU treatment protocol had lower SCEs than those treated with NMU only ([a] $p \leq 0.05$, Duncan's t-test).

1 Address for reprint requests: K.-W. Stahl, Zentrum Biochemie, Abt. I, Medizinische Hochschule, Konstanty-Gutschow-Str. 8, D-3000 Hannover 61 (Federal Republic of Germany).
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Biologically-active sterol sulfates from the marine sponge *Toxadocia zumi*

T. Nakatsu, R.P. Walker, J.E. Thompson and D.J. Faulkner[1,2]

Scripps Institution of Oceanography, La Jolla (California 92093, USA), November 10, 1982

Summary. Three sterol sulfates **1**–**3** having a wide variety of biological activities were isolated from the local marine sponge *Toxadocia zumi*. The structures were determined from spectral data.

In temperate waters, sponges often dominate those assemblages of sessile organisms found on the undersides of rock formations where there is insufficient light for vigorous algal growth. Within these sponge-dominated assemblages, some species are always heavily fouled by epibionts such as hydroids, ectoprocts and algae while other species are remarkably free of epiphytic growth[3]. There does not appear to be any obvious physical rationale for the differences in fouling by epibionts. We have therefore investigated the hypothesis that certain sponges produce biologically active compounds that inhibit the growth of fouling organisms or the settling of their larval forms. Although we recognize that antimicrobial activity may not parallel antifouling activity, it is interesting to note that there is a negative correlation between the antimicrobial activity of sponges and the degree of fouling that they suffer[4].

In order to test the hypothesis that lack of fouling organisms might indicate the presence of biologically-active compounds in a sponge, we chose to study those sponges that are seldom, if ever, fouled and that did not belong to genera usually associated with production of secondary metabolites. *Toxadocia zumi* (Adociidae, Poeciloserina)[5] is such a sponge. In this paper we report the isolation and identification of 3 steroidal sulfates **1**–**3** that have antimicrobial, antifeedant and cytotoxic properties.

Toxadocia zumi was collected at Pt. Loma, San Diego. The freeze-dried sponge was extracted sequentially with hexane, chloroform and methanol and each extract was screened for antimicrobial activity. The methanol-soluble material, the major fraction (3.3% dry weight) from the extraction, was highly active against *Staphylococcus aureus* and *Bacillus subtilis*. A fractionation of the methanol soluble material was performed using Sephadex LH-20 with methanol as eluant, followed by chromatography on silica gel using 6:3:1 chloroform:methanol:ammonia as eluant and finally by reversed phase HPLC on C-18 Partisil-using 30% aqueous methanol as eluant, to obtain 3 steroidal sulfates, 3β-hydroxy-cholest-5,24-dien-19-oic acid sulfate sodium salt (**1**, 0.12% dry weight), 3β-hydroxy-cholest-5-en-19-oic acid sulfate sodium salt (**2**, 0.05% dry weight) and 3β-hydroxy-24-methylene-cholest-5-en-19-oic acid sulfate sodium salt (**3**, 0.05% dry weight) as the major antimicrobial metabolites.

The major sulfate **1** had the molecular formula $C_{27}H_{41}O_6SNa \cdot 2H_2O$ and gave a positive barium nitrate/sodium rhodizonate test for sulfate[6]. The EI mass-spectrum did not contain a molecular ion peak; the highest mass peak was at *m/z* 396 (M-$NaHSO_4$, $C_{27}H_{40}O_2$) with fragmentation peaks at 352 ($C_{26}H_{40}$), 285 ($C_{19}H_{23}O_2$) and 241