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Aliphatic β-Nitroalcohols for Therapeutic Corneoscleral Cross-Linking: Chemical Stability Studies Using ^{1}H-NMR Spectroscopy†

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ABSTRACT

Recent studies suggest that aliphatic β-nitro alcohols may represent a useful class of compounds for use as *in vivo* therapeutic corneoscleral cross-linking agents with higher order nitroalcohols (HONAs) showing enhanced efficacy over the mono-nitroalcohols. The current study was undertaken in order to evaluate the chemical stability of these compounds during storage conditions. Two mono-nitroalcohols (2-nitroethanol=2ne and 2-nitro-1-propanol=2nprop) and two HONAs, a nitrodiol (2-methyl-2-nitro-1,3-propanediol=MNPD), and a nitrotriol (2-hydroxymethyl-2-nitro-1,3-propanediol=HNPD) were monitored for chemical stability by ^{1}H-NMR for up to 7 months. Each compound was studied at two concentrations (1% and 10%) either in unbuffered H_2O or 0.2 M NaH_2PO_4/Na_2HPO_4 (pH=5), and at 0°C and room temperature (RT) for a total of eight conditions for each compound. The ^{1}H-NMR spectra for the starting material were compared to subsequent spectra. Under all four of the conditions studied, both the nitrodiol (MNPD) and nitrotriol (HNPD) were stable for the duration of 7 months. 2nprop became unstable under all conditions at 3 months. 2ne was the most unstable of all the compounds tested. HONAs exhibit excellent chemical stability under long-term storage conditions. In contrast, the nitromonols tested are significantly less stable. These findings are relevant to the translation of this technology into clinical use.

INTRODUCTION

Riboflavin-mediated photochemical stabilization of the cornea (also known as CXL = corneal cross-linking) is an exciting new treatment paradigm in Ophthalmology and is revolutionizing the field of corneal therapeutics. In the short period since its inception in the late 1990s (1), CXL has been proven both effective and safe in stabilizing patients with keratoconus (KC) and post-LASIK keratectasias and is becoming standard of care throughout the world. The cross-linking procedure effectively halts the progression of KC and can be accompanied by an improvement in both corneal curvature (*i.e.* flattening or "normalization" of astigmatism) and/or visual acuity. Briefly, the procedure involves the removal of the outer corneal epithelial layer followed by the application of a 0.1% riboflavin-containing solution to the exposed underlying corneal stromal bed. After confirming adequate infiltration of the riboflavin photosensitizer, a 360 nm emitting light source is used to irradiate the central corneal region for up to 30 min at 3 mW cm^{-2} (2).

Despite these successes, the CXL therapy poses attendant risks and drawbacks related to the use of UVA irradiation, the need for epithelial removal (for riboflavin penetration into the corneal stroma), cytotoxicity, and light source accessibility (for the sclera); and has yet to be approved for clinical use in the US. The long-term effects of UV light exposure to the eye are unclear. UVA irradiation has been implicated in a number of deleterious effects, including lens cortical cataract and retina degeneration (3). Epithelial removal is a painful procedure and increases one's risk for incurring a corneal infection, which has been reported following CXL (4). In addition, because the photochemical cross-linking procedure is cytotoxic to cells, extreme caution is used in treating particularly thin corneas (<400 μm) where the risk of corneal endothelial cell damage is increased, a complication that can lead to detrimental corneal swelling and associated opacification (5). Finally, several research groups, including ours, are exploring the possibility of using chemical cross-linking for scleral stabilization as a means to limit pathologic axial growth in progressive myopia. In this case, a chemical cross-linking approach may be favored over the photochemical method, since administration to the sclera via a sub-Tenon's injection can be performed safely and repeatedly. Previous efforts to apply the riboflavin photochemical approach to scleral cross-linking have been reported. However, several issues not applicable to CXL arise when considering the sclera (particularly the posterior sclera) and include toxicity to the neural retina and accessibility of the UVA light source to the posterior scleral region (6). In favor of this photochemical approach, a single sub-Tenon's injection can diffuse readily throughout the sub-Tenon's space, contacting a wide area of the sclera. Thus, in lieu of such concerns and potential benefits, the development of an alternative cross-linking approach that could avoid the use of UVA light, avoid epithelial removal (for the cornea), is less cytotoxic, and could provide cross-linking to the posterior sclera without requiring a light source, is of significant interest to the field of Ophthalmology. This has prompted a search for candidate chemical cross-linking agents that could be used for therapeutic stabilization of either the cornea and/or sclera.

There are hundreds of cross-linking agents that have been used for a variety of purposes including both industrial as well

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as biomedical applications. The areas of application are vast and there is significant overlap. For example, formaldehyde (7) and glutaraldehyde (8), two of the most widely used cross-linking agents, are used in the production of resins for manufacturing plywood, textiles, etc., as well as for leather-making and rubber-hardening for tires. They have also been used in important biomedical applications such as fixation of bioprostheses (*i.e.* heart valves, skin grafting materials, etc.), biological scaffolds, hydrogels, and tissue fixation for histology/pathology. Some of the many commercially used cross-linking agents include other aldehyde agents such as glyceraldehyde (9), methyl glyoxal (10); the group of carbodiimides (11), genipin (12), imidoesters such as dimethyl adipimidate and dimethyl suberimidate, Denacol-epoxys, derivatives of ethylene glycol dimethacrylate, derivatives of methylenebisacrylamide, divinyl benzene. The lists are seemingly endless.

Although many of these compounds are excellent cross-linking agents for *in vitro* cross-linking applications, the list of available agents is much more limited when considering their possible use as *in vivo* tissue cross-linking agents. Several aspects that are less relevant when considering *in vitro* use, become critical when considering their use in a living being. Such considerations for the cornea include: efficacy under physiologic pH and temperature, permeability, coloration and effects on light transmission, and toxicity. The latter includes cytotoxicity, organismal toxicity, and genotoxicity (*i.e.* mutagenicity/carcinogenicity).

Our recent studies suggest that the class of aliphatic β-nitroalcohols may fulfill many, if not all, of the criteria needed to permit further testing in human clinical trials. Nitroalcohols can function as a formaldehyde delivery system under conditions of physiologic pH and temperature (13). In addition, their small size and water solubility favor permeability (14), have little effects on light transmission (15), and furthermore, although they function to deliver formaldehyde, are considerably less toxic than formaldehyde (16) and test negative in genotoxicity testing (National Toxicology Program [NTP] 2012).

A straight-forward study was carried out over 7 months in order to check the chemical stability of four compounds of potential therapeutic interest. They are two mononitroalcohols, 2-nitroethanol (2ne) and 2-nitro-1-propanol (2nprop), a nitrodiol 2-methyl-2-nitro-1,3-propanediol (MNPD), and a nitrotriol 2-hydroxy-2-nitro-1,3-propanediol (HNPD) (see Chart 1). The conditions of storage used were similar to those found in ophthalmic drop formulations. The results show that the HONAs are stable under the present storage conditions whereas the mono-nitroalcohols are not stable. The experimental setup is described in the following section.

MATERIALS AND METHODS

2-nitroethanol (**2ne**), 2-nitroethane, 2-nitro-1-propanol (**2nprop**), NaH_2PO_4, Na_2HPO_4, deuterium oxide (D_2O), acetone (D6) were all obtained from the Sigma-Aldrich Chemical Co (St. Louis, MO). **MNPD** (MW = 135) and 2-hydroxymethyl-2-nitro-1,3-propanediol (**HNPD**, MW = 151) were obtained from TCI America (Portland, OR). Millipore water (<18.2 MΩ.cm) was used in all the experiments.

Methods explanation: Stability tests by ^1H NMR (400 MHz). Two mono-nitroalcohols (2-nitroethanol=**2ne** and 2-nitro-1-propanol=**2nprop**) and two HONAs, a nitrodiol (2-methyl-2-nitro-1,3-propanediol=**MNPD**), and a nitrotriol (2-hydroxymethyl-2-nitro-1,3-propanediol=**HNPD**) were monitored for chemical stability by ^{1}H-NMR for up to 7 months on either a Bruker 400 or 500 MHz NMR instrument. Each compound was studied at two concentrations (1% and 10%) either in unbuffered H_2O or 0.2 M NaH_2PO_4/Na_2HPO_4 (pH = 5), and either at room temperature (RT) or 0°C for a total of 8 conditions for each compound (see Table 1). Unbuffered solutions of H_2O were noted to maintain pH in the range of 7.0–7.14 throughout the duration of the testing. The ^{1}H-NMR spectra for the starting material were compared to subsequent spectra as monitored at various time points (10 days, 1 months, 3 months, 7 months). All ^{1}H-NMR spectra were recorded in solution. The formation of new peaks over time indicates decomposition of the starting material and hence chemical instability. Similarly, the absence of new peaks indicates chemical stability. No effort was made to identify decomposition products as this was not the intent of the study.

For the unbuffered samples, an aliquot of the storage solution was added to a round bottom flask and H_2O was removed under vacuum via rotary evaporator. The residual was then dissolved in either deuterated water (D_2O) or deuterated acetone ($[CD_3]_2CO$ = acetone-d_6). For pH 5

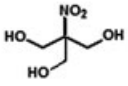
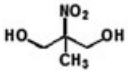
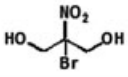
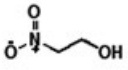
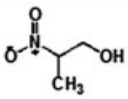
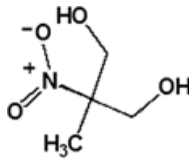
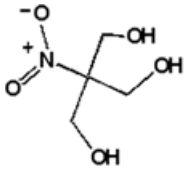
Structure	Chemical Name (CAS)	Abbreviation
	2-hydroxymethyl-2-nitro-1,3-propanediol (126-11-4)	HNPD (nitro-triol)
	2-methyl-2-nitro-1,3-propanediol (77-49-6)	MNPD (nitro-diol)
	2-bromo-2-nitro-1,3-propanediol (52-51-7)	BP
	2-nitroethanol (625-48-9)	2ne
	2-nitro-1-propanol (2902-96-7)	2nprop

Chart 1. The chemical structures of the ß–nitroalcohols studied in this work.

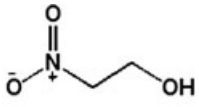
Table 1. Summary of results of stability testing of nitroalcohols.



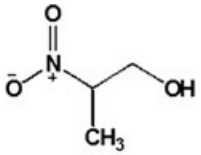
nitro-diol



nitro-triol



nitro-ethanol



nitro-propanol

[C]	Temp	10 days	3 months	7 months	10 day	3 months	7 months	10 day	1 months	10 day	1 months	3 months
1% in H_2O	RT	+	+	+	+	+	+	–	–	+	+	–
	0°C	+	+	+	+	+	+	–	–	+	+	–
10% in H_2O	RT	+	+	+	+	+	+	–	–	+	+	–
	0°C	+	+	+	+	+	+	–	–	+	+	–
1% in pH5	RT	+	+	+	+	+	+	+	–	+	+	–
	0°C	+	+	+	+	+	+	+	–	+	+	–
10% in pH5	RT	+	+	+	+	+	+	+	–	+	+	–
	0°C	+	+	+	+	+	+	+	–	+	+	–

+ denotes stable, – denotes unstable.

buffered samples, an aliquot of the storage solution was first extracted with diethyl ether in order to exclude the buffer. After removing the diethyl ether under vacuum, the residual was dissolved in D_2O or acetone-d_6, in an identical fashion to the unbuffered samples.

As an experimental note, deuterated (deuterium = ^2H, symbolized as D) solvents are preferred for use in ^1H-NMR in order to avoid proton interference coming from the solvent in which the spectra are taken. For example, acetone and H_2O produce peaks at 2.05 and 2.8 ppm, respectively. If these solvents were used for the nitro-diol measurements (see Fig. 1A), peak from these solvents would be present in between the peaks for the nitro-diol protons, which are located at 1.6 and *ca.* 4.0 ppm, complicating the overall spectrum. If, as in this case, D_2O is used as a solvent, the spectrum is much clearer, as shown. Contaminating water from the original sample can also distort the spectrum, and so it is important to fully dry the samples prior to reconstituting in deuterated solvents. Chemical shifts were measured relative to trimethylsilane as previously described in a standard fashion (17). It should be pointed out that the chemical shift for a given compound may differ depending on the solvent condition used.

RESULTS AND DISCUSSION

As nitroalcohol therapeutic corneoscleral cross-linking moves toward use clinically, it becomes increasingly more important to determine compound stability under storage conditions. The optimal method for topical application (to either the cornea or sclera) will be ultimately determined by the degree of effect weighed against any toxicity. For the cornea, the use of these compounds as an eye drop for ongoing daily patient use remains a possibility. However, there are advantages to delivering cross-linking solutions via alternative delivery methods such as a corneal reservoir, hydrogel contact lens, etc. Regardless of the delivery method employed it will be important to have available chemical stability data since in most cases periodic dosing of some form will likely be required in a patient-based setting. Thus, the results of this study have implications for the development of these agents as topical ophthalmic pharmaceuticals.

Nuclear magnetic resonance (NMR) provides an outstanding analytical technique for determining the course of reactions. The starting materials, reactive intermediates and final products can be detected in real time often with minimal interference and with quantification of the species involved. In particular, proton (^1H) NMR is a particularly powerful method since the signals and chemical shifts of the protons in organic molecules appear in distinct regions of the spectrum and the signals are quantitative related to each other by integration. We show below that ^1H NMR can be used as a singlet spectroscopic method to quantitatively determine the stability of nitroalcohols under various conditions of temperature, concentration and pH.

Typical expected ^1H-NMR spectra were identified for each of the compounds tested and were in agreement with existing literature. These are shown as Figs. 1 and 2. As shown in Fig. 1, the spectrum for the nitrodiol **MNPD** shows three sets of proton peaks: the methyl protons shown as a singlet at *ca.* 1.6 ppm, and two doublets at *ca.* 3.85 and 4.1 ppm. The two doublets [(b) in Fig. 1A] are from non-equivalent methylene groups and two protons are coupled with each other forming two doublets. As is the case with the **2nprop** signal (Fig. 2B) because the NMR medium was D_2O, hydrogen bonding and fast proton-deuterium exchange eliminates the hydroxyl proton peak for **MNPD** [(a) in Fig 1A]. The spectrum for the nitrotriol **HNPD** shows two sets of proton peaks for the starting material, one for the hydroxyl protons (n = 3) at *ca.* 4.02 ppm and one for protons of the alpha carbons (n = 6) at *ca.* 4.3 ppm. The hydroxyl proton coupled with the two alpha carbon protons gives a triplet and correspondingly, a doublet is observed for the alpha carbon protons.

As shown in Fig. 2A, three sets of proton peaks for the original **2ne** are seen, as expected, at *ca.* 4.1, 4.38, and 4.6 ppm. Labeled (b) protons at 4.1 ppm are a quartet due to coupling with adjacent methylene protons (c) and the hydroxyl proton (a). The spectrum of **2nprop** (Fig. 2B) either in H_2O or at RT at 10 days was as the original. Peaks are seen at *ca.* 3.95, 4.8, and 4.89 ppm, as well as the 2-methyl group protons seen *ca.* 1.5 ppm. Also, because the NMR medium was D_2O, hydrogen bonding and proton exchange eliminates the hydroxyl proton peak.

Mononitroalcohols become unstable with time

The monols showed instability under these storage conditions. **2ne** was the most unstable of all the compounds tested,

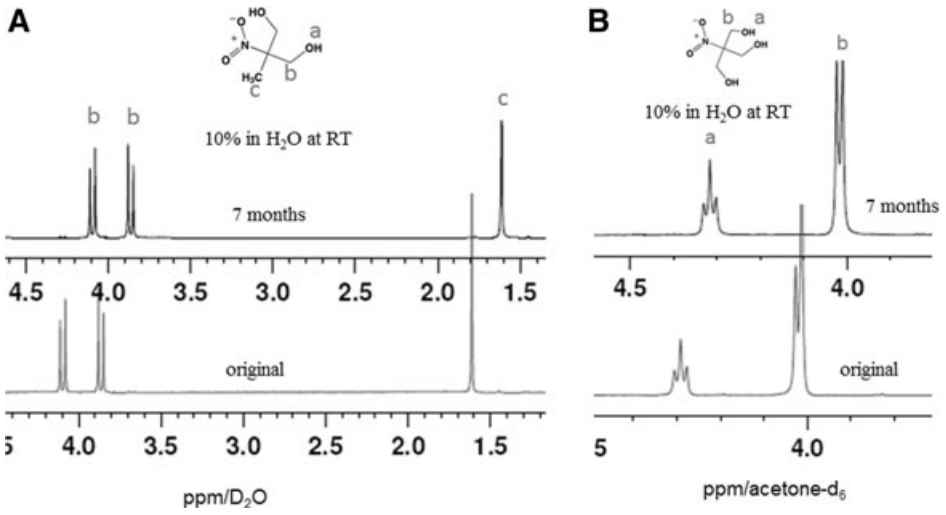


Figure 1. Results of higher order nitroalcohol stability studies [all spectra at 400 MHz]. (A) ^{1}H-NMR spectra are shown for the nitrodiol **MNPD** in D_2O. The three proton peaks (bottom) of original as expected, do not change after 7 months in H_2O at RT (top). Two doublets (b) are from non-equivalent methylene groups. Also, because the NMR medium was D_2O, hydrogen bonding and proton exchange eliminate the hydroxyl proton peak (a) in this spectrum. **MNPD** is stable after 7 months in H_2O at RT. (B) ^{1}H-NMR spectra are shown for nitrotriol **HNPD** in acetone-d_6. The two proton peaks (a) and (b) [bottom spectrum] of original do not change after 7 months in H_2O at RT (top spectrum). Thus, **HNPD** is stable after 7 months in H_2O at RT.

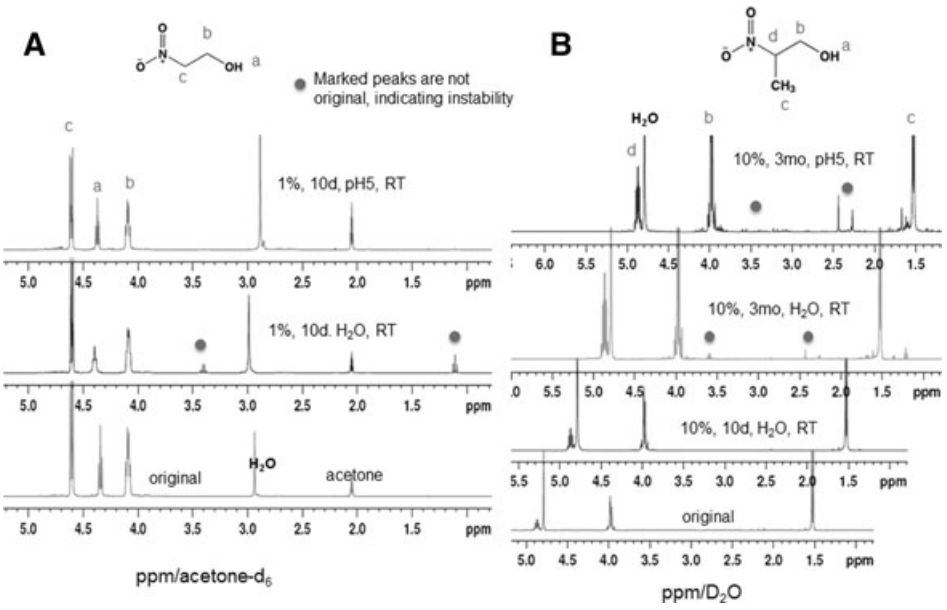


Figure 2. Results of mononitroalcohol stability studies [all spectra at 400 MHz]. (A) ^{1}H-NMR spectra are shown for **2ne** in acetone-d_6. The three proton peaks (bottom) of the original are as expected. In the middle curve of **2ne** in H_2O for 10 days, the peak at 4.35 ppm (a) moved to a less shielded higher frequency field (4.4 ppm). There are two new sets of peaks, a triplet at 1.1 ppm and a quartet at 3.4 ppm with integration 3 and 2, respectively, suggesting that there are $-CH_3$ and $-CH_2$ groups in the decomposition product that couple each other. In pH5 buffer for 10 days, however, the ^{1}H-NMR spectrum (top) is the same as the original. Thus, **2ne** is unstable in H_2O but is stable in pH5 buffer at RT for 10 days. (B) ^{1}H-NMR spectra are shown for **2nprop** in D_2O. The bottom curve belongs to the original sample. The spectrum of **2nprop** in H_2O at RT for 10 days (lower middle) is the same as the original (bottom). However, after 3 months in H_2O and pH5 buffer at RT, the spectra (upper two) changed. There are new peaks observed between 1.5 to 3.9 ppm. Also, because the NMR medium was D_2O, hydrogen bonding and proton exchange eliminates the hydroxyl proton peak (a) in this spectrum. Thus, **2nprop** is stable in H_2O at RT for 10 days but is unstable after 3 months at RT both in H_2O and pH5 buffer.

becoming unstable in unbuffered solution at 10 days. As shown in Fig. 2A in the middle curve for **2ne** in H_2O for 10 days, the peak at 4.35 ppm labeled as (a) has moved to a less shielded higher frequency field (4.4 ppm). In addition, there are two new sets of peaks, a triplet at 1.1 ppm and a quartet at 3.4 ppm with integration 3 and 2, respectively, suggesting that there are $-CH_3$ and $-CH_2$ groups in the decomposition product that couple each other. Sample stability in this case was improved by storage under pH5 acidic conditions but storing in the cold at 0°C did not improve stability. The **2nprop** signal was stable at the

10 day period but became unstable at 3 months. Several new peaks *ca.* 2.2–2.5 ppm and 3.0–4.0 ppm were noted at 3 months under all eight conditions. To reiterate, at 3 months, the **2nprop** instability was not affected by reagent concentration, solvent, or temperature.

In our previous experience, we have noted **a dark reddish-brown** color change to occur during storage of commercially obtained **2ne** and **2nprop** neat solutions. This color change was evident by simple visual inspection. One of the solutions had turned from virtually clear with a slight yellow tint to a dark orange-yellow, indicating the formation of light absorbing impurities. At that time and in response to our inquiry, the chemical supplier carried out a quality assurance QA analysis of our particular reagents. Using gas chromatography with flame ionization detection (GC/FID), the QA analysis identified a **2ne** signal peak change from 97.4% to 86.3% purity. No information on chemical stability from the supplier was known at the time (D. C. Paik, personal communication with Sigma-Aldrich technical support January 2006).

Previous studies using 2-bromo-2-nitro-1,3-propanediol, or Bronopol (BP), suggest likely decomposition mechanisms for nitroalcohols such as those included in this report. The initial decomposition of BP in aqueous alkali is a pH and temperature dependent retro-nitroaldol reaction to give formaldehyde and 2-bromonitroethanol. Reaction of formaldehyde with the starting material produces the nitrotriol HNPD as a major product (18–21). Using 1H-NMR, we confirmed a similar mechanism for 2-nitro-1-propanol which forms the nitrodiol MNPD (16). Secondly, the liberation of nitrite to 35% occurs during BP decomposition and is second-order. This can be accelerated by heating at 100°C in aqueous alkali (22). Consistent with that study, we also found that nitrite is produced during aqueous alkali decomposition of 2ne, 2nprop, MNPD, and HNPD up to a similar extent. Of note, heating in alkali to produce nitrite can be used to measure nitroalcohols in solution by coupling the denitration with a simple colorimetric nitrite assay (23). Finally, it should be mentioned that 2,2-dinitroethanol can also form from BP decomposition and causes a deep reddish-brown color change (24). As pointed out earlier in this results section, both 2ne and 2nprop can form such coloration upon long-term storage raising the possibility that 2,2-dinitroethanol or 2,2-dinitropropanol, respectively, may form.

Importantly, such compound instability may also explain unusual differences in prior results of Ames testing performed on **2ne** (25). Ames testing is routinely performed to determine chemical mutagenicity using various strains of Salmonella typhimurium. The earliest reported result on **2ne** Ames testing was in 1984. In that analysis, **2ne** was found to be equivocal using several common TA strains of Salmonella. However, a repeat analysis in 1988 showed mutagenicity in three strains, including TA98. This particular strain was negative in 1984 but was positive in 1988. Other inconsistencies in the mutagenicity profile were also noted at that time. Communication with the principal investigator (PI) of the study, Dr. Errol Zieger confirmed that the original **2ne** bottle was used for both studies and was stored at RT in the interim. In other words, the **2ne** sample was kept for approximately 4 years between testing dates. In lieu of the contradictory results of the Ames trial and considering our finding of **2ne** instability in solution over just 10 days, it would be reasonable to postulate that the 1988 results could be related to degradation products. Most recently, the NTP under Dr. Christine Witt, performed an extensive battery of high throughput genotoxicity screens on **2ne**. These studies included testing for caspase 8, 9, and 3/7 activation, general cytotoxicity (conducted in 13 different cell types from 5 species), activation of p53 and various additional stress response pathways including heat shock 70 protein and the antioxidant response element, hERG channel interference, disruption of mitochondrial membrane potential, and interaction with a long list of nuclear receptors. These studies also included Ames testing on the TA98 strain which were reported as negative in 1984 and positive in 1988. The current round of NTP Ames testing using the TA98 strain showed no activity when using a freshly prepared/obtained sample of **2ne**. This finding supports the possibility that the 1988 testing was performed on a chemically degraded sample. Furthermore, they have concluded that **2ne** is inactive in all of their testing, confirming absence of any genotoxic effects (D. C. Paik, personal communication, July 2011).

Higher order nitroalcohols remain stable with time

In contrast to the instability of the mononitroalcohols, the HONAs, **MNPD** and **HNPD**, were found to be remarkably stable. At 7 months of storage under various conditions, under all the conditions tested, these two compounds remained stable. In even the most unfriendly conditions for decomposition in our study, unbuffered H_2O at a high 10% concentration kept at room temperature, the compounds were stable. Table 1 summarizes the results of the stability studies of the 4 β-nitro alcohols. It should be pointed out that these conditions are relatively mild by comparison with systematic industry monitored stability testing similar to those undertaken prior to market release.

The World Health Organization has outlined standards for formal systematic pharmaceutical stability testing and shelf-life/re-test period determination (26). Such testing includes temperature storage in 10°C increments, strict humidity control, a simulating container/closure system, pH testing, etc. Testing is conducted on different primary batches of production material. These tests are reserved for compounds well on their way to clinical application. On the other hand an initial study such as ours regarding chemical stability during several different storage conditions provides valuable information at this early stage of drug development. Our results showed that under the selected conditions the HONAs (MNPD and HNPD) remained stable for 7 months while the nitromonols (2ne and 2nprop) were unstable during that time period. We conclude by noting that the HONAs are both more effective and have longer shelf life than the monols. These findings are relevant to translation of this technology into clinical use.

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