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Research paper

Synthesis, characterization and nitric oxide release profile of nitrosylcobalamin: a potential chemotherapeutic agent

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Nitrosylcobalamin, a vitamin B12-based, non-toxic carrier of nitric oxide (NO), has been synthesized, isolated and characterized *in vitro*. A UV/Vis analysis was performed confirming the reduction of the cobalt atom of hydroxocobalamin (vitamin B12a) with the binding of NO, causing a shift in the absorption spectra of Co^{3+} (λ_{max}=530) to Co^{2+} (λ_{max}=500) with the formation of nitrosylcobalamin. The extinction coefficient (ϵ_{max}) of nitrosylcobalamin, as calculated, was 4.8 (mM^{-1}, cm^{-1}). An IR analysis determined the ν(NO) vibrational frequency at 1652 cm^{-1}, supporting the binding of NO and suggesting a bent bonding geometry. NO release was maximized utilizing acidic conditions (pH 4.9, 32°C) with a cumulative release of about 4610 nmol of NO in 675 h (calculated half-life of 210 h), representing 39% NO loading based on 11 890 nmol NO, theoretically possible (one NO per molecule of hydroxocobalamin). The cumulative NO release followed first-order kinetics and was pH dependent. NO release was minimal at pH 6.0 and 7.4 (37°C), and undetected at pH 10 (37°C). The possibility for nitrosylcobalamin to deliver NO (the active chemotherapeutic agent) to neoplastic cells is suggested because tumor cells, specifically leukemia cells, possess surface cell receptors for vitamin B12 which is readily utilized in the cellular proliferation process. Nitrosylcobalamin may offer a 'drug targeting' approach as a potential, biologically compatible and selective chemotherapeutic agent. [© 1998 Rapid Science Ltd.]

Key words: Hydroxocobalamin, leukemia, nitric oxide, nitrosylcobalamin, vitamin B12.

Introduction

When devising chemotherapeutic agents, a major concern is their toxicity to non-target cells. Recent trends in the development of chemotherapeutic agents focus on drug targeting and selective antitumor therapies.[1,2] Of great interest are site-specific carriers, which will deliver a pharmacological agent, capable of destroying targeted cells while avoiding toxicity to surrounding cells. The interactions between vitamin B12 and nitric oxide (NO), a highly reactive free radical, have been widely examined.[3–5] Until this time, vitamin B12 has only been considered as a biological scavenger of NO, overlooking the possibility to implement vitamin B12 as a viable NO donor. NO is an ideal chemotherapeutic agent because intracellular concentrations of NO have been shown to be deleterious to tumor cells [6–9] as well as microorganisms.[10] However, most conventional NO donors, so called NONOates, are not selective in delivering NO, with significant NO release under physiological conditions,[11] thus lacking the selectivity of drug targeting. Another drawback to conventional NONOates is their degradation to non-toxic products which remains questionable.

In devising a NO donor to be utilized as a chemotherapeutic agent, the biological compatiblity of the carrier, after NO release, must be considered, as well as the unique characteristics of the target cells in order to promote selective delivery. A key feature of leukemia cells are surface cell receptors specific for vitamin B12,[12] thus presenting a plausable point of attack in the design of a site-specific chemotherapeutic agent. Vitamin B12 could function in a similar manner to a 'prodrug' releasing NO (the active chemotherapeutic agent) under appropriate conditions while avoiding unwanted toxicity to non-target cells. By designing a vitamin B12-based donor of NO, selective chemotherapy may be a possibility in the destruction of leukemia cells. The objectives of this study are to synthesize a stable, vitamin B12-based NO donor, i.e. nitrosylcobalamin, to determine the conditions which maximize NO release and suggest the theoretical basis for nitrosylcobalamin to be utilized as a potential chemotherapeutic agent.

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Materials and methods

Materials

Hydroxocobalamin (acetate salt) was purchased from Sigma (St Louis, MO). Potassium nitrate (99.999%) and vanadium III chloride (99%) were purchased from Aldrich (Milwaukee, WI). NO was purchased from Matheson Products (Twinsburg, OH). Potassium bromide (IR grade) was purchased from Harshaw Chemical (Solon, OH). Spectrophometric grade methanol was purchased from Baxter Health Care (Burdick and Jackson) (McGaw Park, IL). All other reagent grade materials were purchased from Fisher Scientific (Pittsburgh, PA).

Experimental methods

Nitrosylcobalamin synthesis. Hydroxocobalamin acetate (500 mg) was dissolved in 100 ml of methylene chloride. At room temperature, the sample of hydroxocobalamin was exposed to NO gas for a minimum of 10 days at 100 p.s.i.g. (closed and darkened system) in a manner similar to previously published methods.[13] The system was nitrogen purged of NO gas daily and the samples were re-exposed to fresh NO gas for at least 1 h. The system remained charged at 100 p.s.i.g. after NO exposure. After an observable color change, about 451 mg of a solid product was collected, following rotary evaporation of the methylene chloride, and stored frozen (−6 C) until further analysis.

UV analysis. Samples (5 mg) of hydroxocobalamin and nitrosylcobalamin were dissolved in 10 ml of methanol (spectrophometric grade); concentrations were approximately 0.35 mM. Methanol was used as the blank prior to sample analysis. The samples were analyzed using a HP 8452A Diode Array Spectrophotometer (Hewlett-Packard, Palo Alto, CA). The data were integrated using a HP 89530A MS-DOS software package.

IR analysis. Samples of hydroxocobalamin and nitrosylcobalamin were prepared by adding IR grade potassium bromide in the ratio of 1 mg of the sample to 100 mg KBr and placing them in a IR press at 5000 p.s.i. for 5 min. The resultant pellet was analyzed using a Bomem-Michelson MB-100 Infra-Red spectrophotometer (Bomem, Quebec, Canada). The potassium bromide was stored in a drying oven prior to use.

NO release profile. The kinetic release profile of NO was determined using a Monitor Labs Model 8440 nitrogen oxides analyzer (Monitor Labs, San Diego, CA) as previously described.[13] Three types of buffered solutions were used to independently characterize the release of NO: a 0.1 M acetic acid/acetate buffer (pH 4.9 and 5.0), a 0.1 M phosphate buffer (pH 6.0 and 7.4) and a 0.1 M carbonate/bicarbonate buffer (pH 10.0). Samples of nitrosylcobalamin (16.8 mg: pH 4.9, 32 C to determine NO loading; 5.0 mg: pH 5.0, pH 6.0, pH 7.4, 37 C; 20.6 mg: pH 10.0, 37 C) were placed in 25 ml of the appropriate buffer (degassed with helium) in a closed chamber impinger (wrapped in aluminum foil to maintain darkness) with adjustable valves. At desired intervals the valves were opened whereby the NO released passed into a stream of helium, entered the analyzer and was detected by chemiluminescence[14] to determine NO content. The data output was integrated using an IBM LC/9540 Chromatography Data Integrator (IBM Instruments, Danbury, CT). Standard curves were amended periodically within the period of NO analysis, as needed, using known concentrations of potassium nitrate reacted with vanadium trichloride as previously described,[13] from which to calculate the amount of NO released. The release profile was obtained by plotting the cumulative sum of NO produced (nmol) versus time (h). The release profile followed first-order kinetics (computed using Sigma Plot 4.0; SPSS, Chicago IL) and was determined after regression analysis by plotting the following: ln (maximum release − incremental release) versus time which generated the value of k. The half-life was computed using the equation $t_{1/2} = |0.693/k|$.

Results

The formation of nitrosylcobalamin was initially apparent due to the color change observed with the obtained product. The hydroxocobalamin, as purchased, was dark red in color. Upon exposing the hydroxocobalamin sample to NO, the salt turned a brilliant orange, indicating a reduction of the Co^{3+} of the original hydroxocobalmin to Co^{2+} upon the binding of NO (a free radical electron donor), suggesting the formation of nitrosylcobalamin. The UV/Vis analysis provides evidence for the binding of NO to hydroxocobalamin, demonstrating a reduction of the cobalt of nitrosylcobalamin to (+2) compared with that of the original hydroxocobalamin of (+3). The absorption maximas comparing hydroxocobalamin to nitrosylcobalamin shifted from 278 to 258, 346

to 340 and 530 to 500 (Table 1). The shift in absorption maximas was consistent with experimental results performed by Brouwer *et al.*[4] as well as Rochelle *et al.*[5] comparing Co^{3+} and Co^{2+} in cobalamin-containing compounds. The extinction coefficient of nitrosylcobalamin was calculated to be 4.8 (mM^{-1}, cm^{-1}).

Nitrosylcobalamin was further characterized as to the uptake of NO by comparing 'before and after' IR spectra (Figures 1 and 2). A comparative analysis of the IR spectra provides evidence for the binding of NO to hydroxocobalamin, whereby the axial, hydroxo group (OH) of the original vitamin B12a molecule is displaced by the incoming NO resulting in the formation of nitrosylcobalamin. The ν(NO) of nitrosyl complexes is known to vary from 1900 to 1500 cm^{-1} depending on the nature of the metal, ligand effects, overall molecular charge and other distinctions.[15] The ν(NO) of nitrosylcobalamin occurs at 1652 cm^{-1} with the ν(Co-NO) and δ(CoNO) appearing in the low frequency range of the IR spectrum; however, their assignment requires further interpretation. The proposed geometry of NO binding is most likely bent based on the IR trends observed in related compounds.[15-18] Considering the structural differences of nitrosylcobalamin, the assigned IR bands are consistent, within the 1600 cm^{-1} range, with the IR bands of similar cobalt-containing compounds reacted with NO.[15-18] An unidentified band appears at 1277 cm^{-1}, but may be due to N_2O and NO_2 impurities, normally present in NO gas cylinders,[19] which may have reacted with the hydroxocobalamin molecule.

The NO content and kinetic release profile of nitrosylcobalamin was determined *in vitro* using a nitrogen oxides analyzer (Monitor Labs Model 8440). The amount of NO 'loading' was determined by examining the cumulative NO release, characterized at pH 4.9, 32°C, during a time span of 675.5 h, resulting in the production of 4610 nmol NO (38% loading based on the theoretical maximum of 11 890 nmol: 16.8 mg) (Figure 3). The first 20 h of NO release was characterized in great detail, with data readings taken every 15 min for the first 8 h and every 30 min thereafter, resulting in the production of 260 nmol NO. The cumulative NO release profile followed first-order kinetics (R^2=0.9984, k=0.0033) with a calculated half-life of 210 h. NO release was pH dependent as shown in Figure 3. Samples of 5 mg of nitrosylcobalamin were compared at pH 5.0, 6.0 and 7.4 (37°C) over an 86 h time span producing 95.0, 2.4 and 1.05 nmol NO, respectively. At pH 10.0, NO release was undetetected even though the sample size was increased to 20.6 mg.

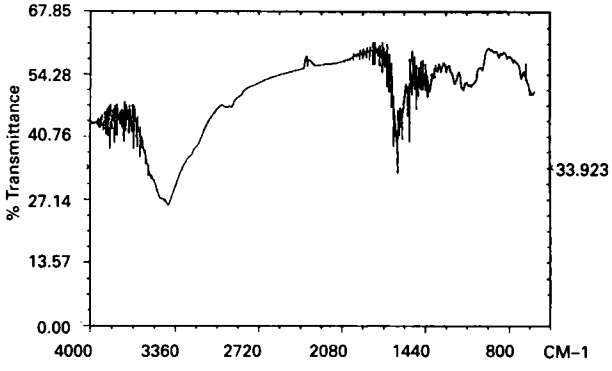

Figure 1. The IR spectra of hydroxocobalamin (KBr pellet).

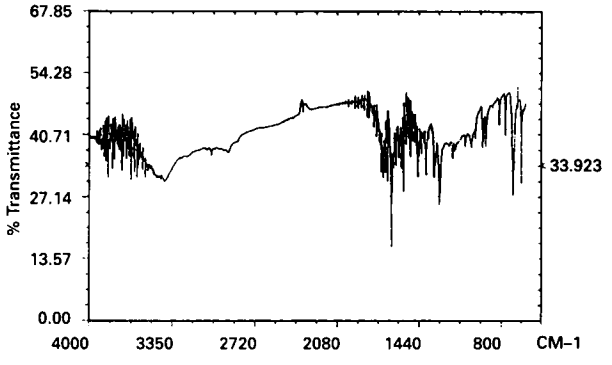

Figure 2. The IR spectra of nitrosylcobalamin (KBr pellet). As NO binds to hydroxocobalamin, the hydroxo group (OH) is displaced. The increase in NO is reflected by an increase in the ν(NO) at 1652 cm^{-1}. The decrease of the hydroxo group is reflected by a decrease in the ν(OH) between 3200 and 3700 cm^{-1}.

Table1. UV/Vis spectral data

Hydroxocobalamin λ_{max} (nm)	Hydroxocobalamin absorbance	Nitrosylcobalamin λ_{max} (nm)	Nitrosylcobalamin absorbance
278	4.132	258	4.132
346	3.910	340	4.099
530	2.302	500	1.694

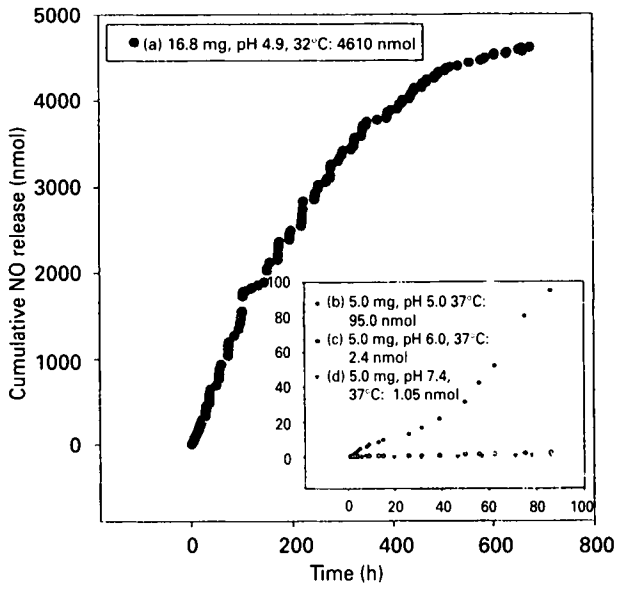

Figure 3. Cumulative NO release profile of nitrosylcobalamin. Samples of nitrosylcobalamin were placed in 25 ml of the appropriate buffer (degassed with helium) in a closed chamber impinger (wrapped in aluminum foil to maintain darkness) with adjustable valves. At desired intervals the valves were opened whereby the NO released passed into a stream of helium, entered a nitrogen oxides analyzer and was detected by chemiluminescence to determine NO content. (a) Total NO release was characterized using 16.8 mg of nitrosylcobalamin, pH 4.9, at 32°C resulting in the production of 4610 nmol of NO during a 675.5 h time period. (b–d) NO release was pH dependent as determined using 5.0 mg samples at 37°C, over an 86 h time period.

Discussion

The specificity some cancer cells have for vitamin B12, especially leukemia cells,[12,20] sets the stage for nitrosylcobalamin to eradicate susceptible cancers which rely on vitamin B12 in the cell proliferation process. As demonstrated by the pH-dependent release profile, the release of NO using nitrosylcobalamin is achieved under acidic conditions (pH 5) with insignificant NO release under physiological conditions (pH 7.4). These conditions correlate well with the acidic conditions (pH 5) encountered in the lysosomal-cellular receptor pathway of B12 target cells [12,21] which provide a specific target to induce NO release. A vitamin B12 donor of NO could function as a 'biological Trojan horse' which would readily be taken into a leukemia cell (via surface cell receptors specific for vitamin B12) whereby NO would be released, resulting in the destruction of the leukemia cell.

Several researchers have shown that N_2O (a less reactive chemical species, in the same family as NO) will reduce the proliferation of leukemia cells.[22,23] Recent evidence suggests the chemotherapeutic effectiveness of cisplatin (an effective anti-cancer drug) may be linked to the production of NO.[24] The damaging effects of NO are explainable as a transition metal phenomena whereby NO has been shown to disrupt primary cellular metabolism by deactivating key transition metal-based enzymes throughout the metabolic cycle.[25–27] Specifically, NO attacks aconitase, an iron-containing enzyme of the TCA cycle, resulting in the shutdown of mitochondrial respiration.[25] Also, NO will completely inactivate the iron-containing enzymes of complex I and II of the electron transport chain.[28] As a result, cellular NAD^+ production ceases thus shutting down glycolysis causing cell death. Furthermore, NO has been shown to decrease levels of intracellular DNA as well as destroy it,[29–31] providing insight into the destructive nature of NO as a cytotoxic agent.

Unlike traditional chemotherapeutic treatments,[1] the use of nitrosylcobalamin suggests a 'biologically friendly' approach to the targeting of both localized and metastasized cancers.

Because nitrosylcobalamin significantly releases NO under acidic conditions, selective anti-tumor therapy may be possible. Intravenous administration of nitrosylcobalmin may be a possibility because of the specificity of NO release under acidic conditions, with minimal release under physiological conditions, whereby toxicity to non-target cells may be avoided. The stability of nitrosylcobalamin under alkaline as well as physiological conditions is significant when examining chemotherapeutic delivery methods which utilize implantable pumps with reservoirs to deliver chemotherapeutic agents.[1] Nitrosylcobalamin can be stored under alkaline or physiological conditions until needed and then pumped into the delivery site whereby a decrease in the pH of target cells, via intracellular lysomes, would cause NO release, resulting in localized delivery.

Conclusion

The results of this study demonstrate the ability of hydroxocobalamin to function as a NO donor compound. The maximal release as occuring at pH 4.9 offers the possibility of selective NO release as compared to most conventional NO donors. This is apparent considering the insignificant release at pH 6.0 and higher. The specificity neoplastic cells have for vitamin B12, although proven in some cases, does not guarantee the targeting of cancerous cells by nitrosylcobalamin but does establish a basis for its potential use as a chemotherapeutic agent. Although the biological compatibility of other NO donors may

be of debate, vitamin B12 which is utilized in the body in a host of physiological processes, is undeniable as to its biocomaptibility thus serving as an ideal donor compound. Considering the chemotherapeutic value of nitrosylcobalamin to neoplastic cells remains to be proven, the selective NO release of nitrosylcobalmin offers a viable foundation on which to direct future studies in the spirit of targeted chemotherapy.

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