Nitric Oxide Scavenging by the Cobalamin Precursor Cobinamide*

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Nitric oxide (NO) is an important signaling molecule, and a number of NO synthesis inhibitors and scavengers have been developed to allow study of NO functions and to reduce excess NO levels in disease states. We showed previously that cobinamide, a cobalamin (vitamin B12) precursor, binds NO with high affinity, and we now evaluated the potential of cobinamide as a NO scavenger in biologic systems. We found that cobinamide reversed NO-stimulated fluid secretion in Drosophila Malpighian tubules, both when applied in the form of a NO donor and when produced intracellularly by nitric oxide synthase. Moreover, feeding flies cobinamide markedly attenuated subsequent NO-induced increases in tubular fluid secretion. Cobinamide was taken up efficiently by cultured rodent cells and prevented NO-induced phosphorylation of the vasodilator-stimulated phosphoprotein VASP both when NO was provided to the cells and when NO was generated intracellularly. Cobinamide appeared to act via scavenging NO because it reduced nitrite and nitrate concentrations in both the fly and mammalian cell systems, and it did not interfere with cGMP-induced phosphorylation of VASP. In rodent and human cells, cobinamide exhibited toxicity at concentrations ≥50 μM with toxicity completely prevented by providing equimolar amounts of cobalamin. Combining cobinamide with cobalamin had no effect on the ability of cobinamide to scavenge NO. Cobinamide did not inhibit the in vitro activity of either of the two mammalian cobalamin-dependent enzymes, methionine synthase or methylmalonyl-coenzyme A mutase; however, it did inhibit the in vivo activities of the enzymes in the absence, but not presence, of cobalamin, suggesting that cobinamide toxicity was secondary to interference with cobalamin metabolism. As part of these studies, we developed a facile method for producing and purifying cobinamide. We conclude that cobinamide is an effective intra- and extracellular NO scavenger whose modest toxicity can be eliminated by cobalamin.

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§ The abbreviations used are: NO, nitric oxide; BHK, baby hamster kidney cells; Delta-NONOate, (Z)-1-[1-(Z)-2-aminoethyl]-N-(Z)-2-ammonio-ethyl]amino]diazen-1-um-1,2-diolate; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; G-kinase, cGMP-dependent protein kinase; HPLC, high performance liquid chromatography; LPS, lipopolysaccharide; NOS, nitric-oxide synthase; O2-Cbl, hydroxocobalamin; PAPA-NONOate, (Z)-1-[(Z)-3-ammoniopropyl]-N-(Z)-propylamino]diazen-1-um-1,2-diolate; 8-PTC-GMP, 8-parachlorophenylythiocyclic GMP; VASP, vasodilator-stimulated phosphoprotein; VSV, vesicular stomatitis virus.

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Cobinamide Is a NO Scavenger

Efficacy of Cobinamide as a NO Scavenger in a Drosophila Model—The standard method for producing diaquocobinamide starts with dicyanocobinamide, removing the cyanide by acid treatment, and exposure to strong light (23). As mentioned earlier, cobinamide has a very high binding affinity for cyanide, and hence it is difficult to remove cyanide completely from the cobinamide preparation. Moreover, exposure to light over a prolonged period can potentially alter the corrin ring. Because we were interested in producing cyanide-free cobinamide for use in biological systems, we started with OH-Cbl as the initial substrate. The dimethylbenzimidazole ribonucleotide tail was removed by brief acid treatment, and the diaquocobinamide was purified by batch elution over a small sample preparation column as described under “Experimental Procedures.” Beginning with about 200 mg of OH-Cbl, we obtained ~150–170 mg of high purity cobinamide. Fig. 2 shows the absorbance spectrum of a typical cobinamide preparation at pH 3 having a major peak of 348 nm and smaller relatively equal peaks at 494 and 520 nm (23, 24); in preparations containing contaminants, the 494 and 520 nm peaks tend to either merge together into one broad peak, or the 520 peak became predominant, and a broad band at 455 nm became evident (25). Further evidence for high purity of the preparations was that at pH 12 the $A_{455}/A_{395}$ ratio was 1.06, well within the range of 1.05–1.11 reported previously for pure dihydroxocobinamide (25), and HPLC analyses of the cobinamide product yielded a single peak when monitored at multiple wavelengths between 300 and 600 nm (40).

Production and Analysis of Cobinamide—The standard method for producing diaquocobinamide starts with dicyanocobinamide, removing the cyanide by acid treatment, and exposure to strong light (23). As mentioned earlier, cobinamide has a very high binding affinity for cyanide, and hence it is difficult to remove cyanide completely from the cobinamide preparation. Moreover, exposure to light over a prolonged period can potentially alter the corrin ring. Because we were interested in producing cyanide-free cobinamide for use in biological systems, we started with OH-Cbl as the initial substrate. The dimethylbenzimidazole ribonucleotide tail was removed by brief acid treatment, and the diaquocobinamide was purified by batch elution over a small sample preparation column as described under “Experimental Procedures.” Beginning with about 200 mg of OH-Cbl, we obtained ~150–170 mg of high purity cobinamide. Fig. 2 shows the absorbance spectrum of a typical cobinamide preparation at pH 3 having a major peak of 348 nm and smaller relatively equal peaks at 494 and 520 nm (23, 24); in preparations containing contaminants, the 494 and 520 nm peaks tend to either merge together into one broad peak, or the 520 peak became predominant, and a broad band at 455 nm became evident (25). Further evidence for high purity of the preparations was that at pH 12 the $A_{455}/A_{395}$ ratio was 1.06, well within the range of 1.05–1.11 reported previously for pure dihydroxocobinamide (25), and HPLC analyses of the cobinamide product yielded a single peak when monitored at multiple wavelengths between 300 and 600 nm (40).

Efficacy of Cobinamide as a NO Scavenger in a Drosophila Fluid Secretion Model—It has become clear that Drosophila is an excellent model for human disease and drug discovery (41, 42). The Malpighian tubules of D. melanogaster are the organs for fluid transport and osmoregulation in the insect, corresponding to vertebrate kidneys. Rates of tubular secretion can be measured in vitro after extracting tubules from flies; NO stimulates secretion via activation of soluble guanylate cyclase, thereby increasing the intracellular cGMP concentration and activating the cGMP/G-kinase transduction pathway (27, 28). We studied the effect of cobinamide on tubular fluid secretion stimulated both by a NO donor and by LPS, an inducer of the Drosophila NOS gene. In addition, to simulate conditions in a whole animal, we administered cobinamide to the flies via their food and then measured the effect of LPS on rates of tubular fluid secretion.

In the experiments with a NO donor, we treated tubules with 10 μM Deta-NONOate, which caused a rapid and sustained increase in the rate of fluid secretion (Fig. 3A; Deta-NONOate was added to all tubules after a 30-min basal period, as indicated by the arrow). Adding 10 μM cobinamide to Deta-NONOate-treated tubules reduced the rate of fluid secretion significantly, almost returning to the basal unstimulated level (Fig. 3A; cobinamide was added at 60 min to some of the tubules as indicated by the arrow and the filled circles). Adding cobinamide alone to the tubules was without effect (data not shown). To increase endogenous NO production by the tubules, we used LPS and observed a marked stimulation of tubular secretion (Fig. 3B; LPS was added at 30 min as indicated by the arrowhead). As in the experiments with Deta-NONOate, cobinamide rapidly reduced tubular secretion, returning rates near to the basal state (Fig. 3B; cobinamide was added at 60 min to some of the tubules as indicated by the arrow and the filled circles). Thus, cobinamide scavenges both extracellularly administered and intracellularly produced NO in a Drosophila whole organ system.

As part of these studies, we measured the amount of NO released by Deta-NONOate or produced by LPS-treated tubules by incubating tubules for 60 min in Schneider’s medium and measuring the sum of nitrite and nitrate in the medium. In the absence of the two drugs, no nitrite or nitrate could be
Cobinamide at a final concentration of 10 mM was added to some of the tubules at 60 min (drug addition noted by arrowhead). Cobinamide at a final concentration of 10 mM was added to some of the tubules at 60 min (drug addition noted by arrowhead in both A and B, and by the filled circles); some tubules received 10 mM cobinamide plus 10 mM cobalamin (filled triangles in both A and B). Each data point represents the mean ± S.D. of at least three independent experiments performed on 20 pairs of Malpighian tubules. C, 10 pairs of tubules were incubated in 200 µl of Schneider’s medium for 60 min with either 10 mM Deta-NONOate or 10 mM LPS in the absence (open bars) or presence (filled bars) of 10 mM cobinamide (Cbi). Nitrite and nitrate in the medium were measured by a Griess reagent-based method and were quantified by comparison with standards. The data are the mean ± S.D. of at least two independent experiments performed in triplicate; the y axis on the left is for the Deta-NONOate data, and the y axis on the right is for the LPS data. No nitrite or nitrate could be detected in the medium from untreated tubules.

detected in the medium, implying that the basal level of NO production by the tubules was very low. Treating the tubules with 1, 10, or 100 mM Deta-NONOate increased the nitrite and nitrate concentrations in the medium to levels above the starting concentrations of the drug, which was likely because Deta-NONOate releases 2 mol of NO/mol of parent compound (Fig. 3C shows data for 10 mM Deta-NONOate). The tubules were probably exposed to somewhat lower NO concentrations, because as mentioned previously, Deta-NONOate will continue to release NO during the measurement of nitrite and nitrate. Treating the tubules with 10 mM LPS to induce NOS levels also increased medium nitrite and nitrate concentrations, but the levels were less than in the tubules treated with 10 mM Deta-NONOate (Fig. 3C). In both the Deta-NONOate- and LPS-treated tubules, cobinamide reduced the amount of combined nitrite and nitrate by about 50% (Fig. 3C), which is consistent with our previous work showing that each cobinamide molecule can neutralize two NO molecules, converting one to nitrite and binding the second one (22); thus, a maximal 50% reduction in nitrite and nitrate could be expected.

To determine whether systemically administered cobinamide would affect rates of tubular fluid secretion, we fed the flies food containing 250 µM cobinamide for 2 days; at this concentration, cobinamide had no apparent detrimental effect on the flies. LPS-stimulated secretion rates of Malpighian tubules were compared between cobinamide-fed flies and flies fed normal control food. We found that tubular secretion rates were reduced by about 50% in the cobinamide-fed flies compared with control flies (Fig. 4). In addition to providing further evidence for the effectiveness of cobinamide as a NO scavenger, these data indicate that cobinamide was absorbed through the gut of the flies and was transported to the Malpighian tubules.

Cobinamide Uptake by Mammalian Cells—Some NO scavengers, such as hemoglobin, are solely extracellular and neutralize only that NO that diffuses out of cells. The studies in Drosophila indicated that cobinamide was likely taken up by the cells, but insect and mammalian transport systems may differ. To determine whether cobinamide has access to intracellular NO in mammalian cells, we produced [14C]dicyanocobinamide and studied its uptake in BHK cells. We found a progressive curve-linear increase in radioactivity in BHK cells over a 1,000-fold concentration range of dicyanocobinamide from 100 nM to 100 µM, suggesting that both passive diffusion and active transport of the dicyanocobinamide were occurring. Because of the progressive increase in cellular radioactivity, a $K_m$ could not be calculated. Although it is possible that the
Cobinamide was not actually taken up by the cells, but rather just bound to a surface receptor, this seems unlikely because of the continued increased in radioactivity over a three log scale of dicyanocobinamide concentrations. Thus, these data suggest that cobinamide has the potential to serve as both an intra- and extracellular NO scavenger in mammalian systems.

Efficacy of Cobinamide as a NO Scavenger in Mammalian Cells—To determine whether cobinamide can serve as a NO scavenger in mammalian cells, we examined NO stimulation of VASP phosphorylation in two different types of cultured cells. VASP is an important regulator of actin dynamics and thus of cellular processes such as cell adhesion and motility (31). Its function is regulated by phosphorylation, and NO, via activation of the cGMP/G-kinase transduction pathway, is a major inducer of VASP phosphorylation. To study the effect of cobinamide on NO-induced phosphorylation of VASP, we chose rat C6 glioma cells and CS-54 vascular smooth muscle cells, both of which have an active NO/cGMP transduction pathway (34, 43).

In C6 cells we studied the effect of cobinamide on exogenously generated NO, and in CS-54 cells we studied the effect of cobinamide on endogenously produced NO.

When C6 cells were treated for 30 min with 15–60 μM PAPA-NONOate, VASP phosphorylation was induced, as evidenced by generation of a VASP form with reduced electrophoretic mobility (Fig. 5A, compare first lane, no PAPA-NONOate, to third, fifth, and seventh lanes, showing 15, 30, and 60 μM PAPA-NONOate, respectively). At all PAPA-NONOate concentrations, adding 100 μM cobinamide to the culture medium prevented the increase in VASP phosphorylation (Fig. 5A, compare third, fifth, and seventh lanes with fourth, sixth, and eighth lanes showing cells treated with cobinamide and PAPA-NONOate). Human hemoglobin (100 μM) yielded results similar to those with cobinamide, indicating that under the experimental conditions, cobinamide and hemoglobin were equally effective NO scavengers. Cobinamide also prevented increases in VASP phosphorylation when used at an equimolar concentration as PAPA-NONOate (Fig. 5B shows 30 μM each PAPA-NONOate and cobinamide, but similar results were also found at 15 and 60 μM). As discussed further below, PAPA-NONOate releases 2 mol of NO and thus as in the Drosophila Malpighian tubules, each cobinamide molecule appeared to neutralize two NO molecules.

Because it was possible that cobinamide could be inhibiting the NO stimulation of VASP phosphorylation through some mechanism other than NO scavenging, we studied the effects of cobinamide on cGMP stimulation of VASP phosphorylation. We used the membrane-permeable cGMP analog 8-pCPT-cGMP at a concentration of 30 μM to induce VASP phosphorylation and found no effect of cobinamide at concentrations from 30 to 100 μM (Fig. 5C, compare second lane, CPT-cGMP alone, with third lane, CPT-cGMP plus cobinamide). Thus, cobinamide did not interfere with the activation of G-kinase or the phosphorylation of VASP, and thus, it appeared to be acting via NO scavenging.

Because calcium activates types I and III NOS, we tested CS-54 cells with the calcium ionophore A23187 to increase endogenous NO production. We found that A23187 increased VASP phosphorylation (Fig. 5D, compare fourth lane with first lane) and that cobinamide significantly attenuated this effect (Fig. 5D, compare third with fourth lane). Thus, cobinamide is an effective intra- and extracellular NO scavenger in mammalian cells.

As in the studies of the Malpighian tubules, we measured nitrite and nitrate concentrations in the culture medium of the C6 and CS-54 cells. As mentioned above, PAPA-NONOate, like DETA-NONOate, releases two NO molecules/mol of parent compound, and we found that treating C6 cells for 30 min with 15 μM PAPA-NONOate increased the concentration of nitrite and nitrate in the medium to almost 30 μM (when compared with untreated cells, Fig. 5E). Because of the short half-life of PAPA-NONOate, 15 min at 37 °C, the vast majority of the NO was released during the incubation period and not during the sub-
sequent measurement of nitrite and nitrate. Treating CS-54 cells with 300 nM A23187 increased nitrate and nitrite concentrations in the medium to about 12 μM (Fig. 5E). When 15 μM cobinamide was added to the PAPA-NONOate-treated C6 cells or the A23187-treated CS-54 cells, there was a significant decrease in the nitrite plus nitrate concentrations, with a larger effect in the CS-54 cells than in the C6 cells (Fig. 5E). Thus, the cobinamide-induced decrease in VASP phosphorylation in the two cell types was reflected by a reduction in nitrite and nitrate, providing further evidence that cobinamide was functioning as a NO scavenger in reducing VASP phosphorylation.

Studies of Cobinamide Cytotoxicity—As a cobalamin analog, cobinamide could be cytotoxic by interfering with cobalamin metabolism or function. Moreover, as a NO scavenger, cobinamide could interfere with the proliferative and antiapoptotic actions of NO in some cell types (44, 45). To study the potential toxicity of cobinamide in mammalian cells, we performed several sets of experiments. First, we assessed the effect of cobinamide on the growth of BHK, C6, and CS-54 cells, as well as two primary human cell lines, i.e. foreskin fibroblasts and human umbilical venous endothelial cells; the latter cells were included to determine whether there was differential toxicity of cobinamide to primary cells than to established cell lines. We found that at concentrations between 1 and 50 μM, cobinamide had no effect on cell growth, but at concentrations ≥50 μM, cobinamide inhibited the growth of all five cell types, albeit by a minimal amount at 50 μM (Fig. 6, data are shown at cobinamide concentrations of 50, 100, and 200 μM in BHK cells (main figure) and C6 and CS-54 cells (inset); similar results were found in the two primary cell lines). Growth inhibition was reversed completely by an equimolar concentration of cobalamin at all three cobinamide concentrations in all five cell types (Fig. 6; data are shown only for 200 μM cobinamide/cobalamin in BHK cells (main figure)). These latter data suggest that the mechanism of toxicity was through competitive interference of cobalamin metabolism or function rather than through NO scavenging. Thus, cobinamide was toxic to cells at only relatively high concentrations, and its toxicity could be reversed fully by cobalamin.

The next set of experiments was directed at determining whether cobinamide inhibited the activities of either methionine synthase or methylmalonyl-CoA mutase, the two vitamin B12-dependent enzymes in mammalian cells. At concentrations as high as 200 μM, we found no effect of cobinamide on the activities of either enzyme as measured in BHK cell extracts. Methionine synthase was assumed to be in the holoenzyme form (46), and methylmalonyl-CoA mutase apoenzyme was converted to holoenzyme by incubation with 5 μM deoxyadenosylcobalamin followed by a 10-fold dilution of the extract as described under “Experimental Procedures.” Thus, at the highest concentration of cobinamide tested, the cobinamide concentration was 400 times that of the deoxyadenosylcobalamin concentration. The lack of inhibition of either enzyme by cobinamide is consistent with studies reported by others on various cobalamin analogs including cobinamide (47, 48).

In the final set of experiments, we assessed in BHK cells the effect of cobinamide on the in vivo activities of methionine synthase and methylmalonyl-CoA mutase by following the incorporation of [14C]formate into purine nucleotides and [14C]propionic acid into protein; the former assay is a measure of carbon flux through the folate pathway and is dependent on methionine synthase activity, whereas the latter assay is dependent on methylmalonyl-CoA mutase activity. In both assays, we found that 100 μM cobinamide decreased incorporation of the radioactive label by about 50% (Fig. 7; filled bars are data for [14C]formate incorporation, and open bars are data for [14C]propionic acid incorporation). The inhibition of enzyme activities was time-dependent, and in the case of methionine synthase, minimal inhibition was observed after 90 min of cobinamide exposure, the shortest time point that could be measured; inhibition increased progressively with time, and the data in Fig. 7 are for a total of 8 h of cobinamide exposure.
Similar time dependence of enzyme inhibition was observed for methylmalonyl-CoA mutase, but over a longer time scale. As in the growth studies, the toxic effect of cobinamide was prevented completely by an equimolar concentration of cobalamin (Fig. 7). These latter data indicate that the mechanism of cobinamide toxicity is likely through interference with some aspect of cobalamin metabolism (as considered further under “Discussion”). Moreover, because methionine synthase and methylmalonyl-CoA mutase are the only two mammalian cobalamin-dependent enzymes, the cobalamin reversal data suggest that the [14C]formate and [14C]propionic acid incorporation assays accurately reflect the in vivo activity of these two enzymes.

**Efficacy of Cobinamide as a NO Scavenger in the Presence of Cobalamin**—Although cobinamide is likely to be used at concentrations where it is not toxic, it would be useful to know whether cobinamide and cobalamin can be used together when scavenging NO. Cobalamin itself is a weak NO scavenger (21) and would not be expected to interfere with the cobalamin scavenging of NO. We found that OH-Cbl had no effect on NO scavenging by cobinamide, either on tubular fluid secretion in *Drosophila* Malpighian tubules (Fig. 3, A and B, compare filled circles, cobinamide alone, with filled triangles, cobinamide plus cobalamin) or on VASP phosphorylation in mammalian cells (Fig. 5B, compare fourth lane, cobinamide alone, with fifth lane, cobinamide plus cobalamin). The combination of cobinamide plus cobalamin, like cobinamide alone, had no effect on VASP phosphorylation induced by 8-pCPT-cGMP (Fig. 5C, compare third and fourth lanes).

**DISCUSSION**

Since the original description of NO as endothelium-derived relaxation factor in 1978, it has become clear that NO has many physiological roles (1–3). It also has become clear that NO contributes to the pathophysiology of several disease states including septic and hemorrhagic shock, hepatic encephalopathy, hepatorenal syndrome, hemodialysis-related hypotension, and ischemia-reperfusion injury (5–7). In most of these diseases, abnormally high NO production induces profound vasodilation and vasopressor-refractory hypotension. A marked increase in iNOS underlies the elevated NO production in sepsis (49), and nonselective NOS inhibitors increase blood pressure in animal models of sepsis, but have had a mixed effect on sepsis-associated mortality in animals and humans (18, 50); this may relate, in part, to inhibition of eNOS leading to microvascular vasoconstriction and decreased tissue and organ perfusion (51–55). Selective iNOS inhibitors should avoid some of the problems encountered with nonspecific inhibitors, but iNOS expression in some tissues may be functionally important as has been found for myocyte iNOS (11, 56–59). Thus, even selective iNOS inhibition may lead to dysfunctional changes in cells and organs, and several groups of workers have urged caution in using any type of NOS inhibitor in sepsis (18, 50). This failure may relate, in part, to inhibition of eNOS leading to microvascular vasoconstriction.

There is a clear need, therefore, for agents that can lower NO concentrations up to 100 μM before exhibiting significant toxicity. Other than during pharmacological administration of NO, it is unlikely that NO concentrations ever exceed 10 μM under physiological conditions (7, 10, 11). Moreover, we found that cobalamin completely prevented cobinamide toxicity and that cobalamin did not interfere with NO scavenging by cobinamide, either in the *Drosophila* Malpighian tubule secretion model or in mammalian cells; as mentioned previously, cobinamide itself is a NO scavenger (21).

At the relatively high concentrations where cobinamide began to exhibit toxicity, it appeared to interfere with cobalamin metabolism or function. This conclusion is based on the findings that cobinamide had no effect on the in vitro activities of methionine synthase and methylmalonyl-CoA mutase, the two mammalian cobalamin-dependent enzymes, whereas it inhibited in vivo assays of the enzymes in a time-dependent and cobalamin-reversible fashion. In the in vitro assays, the enzymes were in the cobalamin-containing holoenzyme form, indicating that cobalamin cannot compete with the bound cofactor. Other workers have also found no inhibition in vitro of the holoenzyme form of methionine synthase by cobalamin analogs, and in fact, cobinamide can restore the methionine synthase apoenzyme to full activity; but whether this occurs in vivo is not known (47). The effect of cobalamin analogs on methylmalonyl-CoA mutase activity has apparently not been studied, but 5'-deoxyadenosycobinamide is inactive in restoring function to the apoenzyme of methylmalonyl-CoA mutase.

**2** V. Singh, V. S. Sharma, and G. R. Boss, unpublished observations.

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The time-dependent inhibition of enzyme activity in the in vivo assays occurred over hours, suggesting that newly synthesized enzyme was inhibited; the half-life of methionine synthase is about 12 h and that of methylmalonyl-CoA mutase appears to be ~30 h (46, 75). Thus, cobinamide could interfere with incorporation of methylcobalamin or deoxyadenosylcobalamin into newly synthesized methionine synthase and methylmalonyl-CoA mutase, respectively, or perhaps more likely, with the cellular transport of OH-Cbl or its conversion to the two coenzyme forms. Similar conclusions about the mechanism of toxicity of cobalamin analogs were reached previously (47). Whatever the precise mechanism(s) of cobinamide cytotoxicity is, it can be completely prevented by coadministration of cobalamin.

Because of its high binding affinity for cyanide, cobinamide could also be expected to be an excellent cyanide scavenger and could potentially be used in clinical states of cyanide toxicity, could also be expected to be an excellent cyanide scavenger and of cobalamin. Whatever the precise mechanism(s) of cobinamide cytotoxicity of toxicity of cobalamin analogs were reached previously (47). The cellular transport of OH-Cbl or its conversion to the ymalonyl-CoA mutase, respectively, or perhaps more likely, with incorporation of methylcobalamin or deoxyadenosylcobalamin and could be combined with cobalamin.

In conclusion, cobinamide is an effective NO scavenger in Drosophila Malpighian tubules and cultured mammalian cells and is likely to be useful in animal studies. It may also be beneficial in clinical states of excess NO and in cyanide toxicity and could be combined with cobalamin.

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