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A series of six biochemical markers of cyanide toxicity (dopamine release, peroxide generation, cytosolic-free calcium, catalase activity, cytochrome · oxidase activity and superoxide dismutase activity) in cultured rat· pheochromocytoma (PC12) cells were used to establish a screen for evaluation of potential anticyanide compounds. Thirty-nine substances, including anticonvulsants, adrenergic blockers, antioxidants, antipsychotics, etc., were tested and ranked according to the results.

Based on the composite scoring in all six assays, carbamazepine, mannitol, allopurinol and phenytoin were ranked as the most effective anticyanide compounds. Additionally, known cyanide antidotes (e.g., pyruvate, mercaptopyruvate, alpha ketoglutarate, naloxone and

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19. continued

flunarizine) obtained relatively high ranking in the PC12 cell screen. Furthermore, a significant correlation was found between protective effects (based on LD_{50}s) of cyanide antidotes in mice and ranking in the in vitro screen. This study illustrates that by assaying a series of biochemical markers in a neuron-cell line, a rapid, cost-effective in vitro toxicological screen for cyanide antidotes is possible. Several compounds have been identified which inhibit the biochemical effects of cyanide and may be used to enhance effectiveness of the standard cyanide antidotes.

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INTRODUCTION

Standard antidotes for cyanide (sodium thiosulfate and sodium nitrate) have been used since 1934 (Chen *et al.*). The long accepted mechanism of action for thiosulfate is that it serves as a sulfane-sulfur donor, in the presence of the mitochondrial enzyme rhodanese, to form the comparatively nontoxic and excretable thiocyanate. Sodium nitrite generates methemoglobin which combines with and inactivates cyanide. However many biochemical actions of cyanide have only recently been characterized (Johnson *et al.*, 1986, 1987a; Maduh *et al.*, 1988). The goal of this work was to develop an <u>in</u> <u>vitro</u> screen for substances which inhibit biochemical alterations caused by cyanide. For example, an increase in free intracellular calcium occurs in neuronal cells exposed to cyanide and this change appears to be independent of any effect on energy metabolism (Johnson *et al.*, 1986, 1987a; Maduh *et al.*, 1988, 1991). The goal of this work was to develop an <u>in</u> <u>vitro</u> screen for substances which inhibit biochemical alterations caused by cyanide. These substances may then be used to enhance the antidotal effectiveness of the thiosulfate-nitrate treatment.

Since the brain is a major target for cyanide, biochemical changes produced by cyanide were evaluated in cultured neuron-like cells ("PC12" cells from a rat adrenomedullary tumor). The PC12 cell line originally established by Greene and Tischler (1976) is a well characterized transformed cell line which exhibits numerous characteristics of sympathetic neurons and can be used to evaluate neurochemical markers of toxicity (Johnson et

al., 1986, 1987a; Maduh et al., 1990; Shafer and Atchison, 1991). The cells express functional calcium channels and membrane-bound receptors, and are electrically excitable (Shafer and Atchison, 1991). Following stimulation, the cells secrete dopamine by a calcium dependent process (Kanthasamy et al., 1991b). This cell line is an excellent in vitro model for toxicity testing, and the present study illustrates the use of these cells to screen for anticyanide compounds.

Chemicals screened as potential antidotes included anticonvulsants, adrenergic blockers, antiarrhythmics, antioxidants, carbohydrates and antipsychotics. Known cyanide antidotes were also used for comparative purposes and to allow a correlation to be drawn between in vitro effectiveness and antidotal efficacy in the mouse LD_{50}.

MATERIALS AND METHODS

Cell Culture

PC12 cells were obtained from American Type Culture Collection (Rockville, MD) and grown as monolayers in RPMI 1640 media (85% v/v) supplemented with 10% v/v heat-inactivated horse serum, 5% v/v fetal bovine serum and penicillin, 5000 U/ml, and streptomycin, 50 μg/ml, in plastic tissue culture flasks. Inactivation of horse serum was effected by heating at 56°C for 30 min. Cells cultured for 5 to 6 days after transfer were harvested from monolayers and suspended in Krebs-Ringer bicarbonate (KRB) solution (125 mM NaCl, 5 mM KCl, 25 mM HEPES-NaOH, 6 mM glucose, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and

1 mM CaCl$_2$) at pH 7.4. Cell populations were estimated by counting and appropriate dilutions were made.

Pretreatments

All potential antidotes (10 μM except for thiosulfate, which was also used at a 100 μM concentration) were added to suspensions of PC12 cells 15 min prior to cyanide. The concentrations of cyanide employed (range 0.05 to 5 mM) were determined from dose-response curves for each individual assay. Results obtained with cyanide alone were compared with those obtained using cyanide plus the compound being screened. Controls were run to determine basal activity of each biochemical marker. Enzyme activity was determined in cell homogenates following 30 min incubation with cyanide. Experiments were generally repeated three to five times to establish the validity of the observations.

Antioxidant Defense Enzymes

Catalase: Hydrogen peroxide is damaging to living systems, generally because it gives rise to OH$^{\bullet}$ radicals which can produce alterations in DNA, lipids and proteins in cells. It is therefore important that cells limit H$_2$O$_2$ accumulation. Catalase mediates the conversion of H$_2$O$_2$ to H$_2$O and O$_2$. The enzyme is distributed throughout the body and may play an important role in limiting oxidative damage after cyanide exposure.

Enzyme activity was measured at 20°C in 50 mM sodium phosphate buffer at pH 7.0 using 50 mM H$_2$O$_2$ as substrate (Aebi, 1974). After incubation of cells for 30 min with potassium cyanide (KCN), decomposition of H$_2$O$_2$ by catalase was monitored by

measuring the change in extinction spectrophotometrically (Perkin Elmer Lambda 3B) at 240 nm for 3 min. Catalase activity was determined as a first-order rate constant (k). Values were expressed as percent control. Potential antidotes were tested against an IC_{50} concentration of KCN (55 μM).

Superoxide Dismutase (SOD): CuZn superoxide dismutase, which is the cyanide-sensitive form, is found in virtually all mammalian cells and serves to catalyze breakdown of superoxide (O_2^-) to H_2O_2 and O_2. This reaction is important because it prevents interaction of O_2^- with iron in the cell and blocks formation of the highly reactive hydroxyl radical $OH^{\bullet}$ (Halliwell, 1987).

SOD enzyme levels were determined spectrophotometrically (Perkin Elmer Lambda 3B) at 560 nm by the nitro blue tetrazolium method in which total SOD activity is estimated in homogenates (Oberly and Spitz, 1985). Samples were incubated with cyanide for 30 min before determination of SOD activity, which was measured as amount of enzyme (1 unit) that inhibits reduction of nitro blue tetrazolium by 50%. Values were expressed as percent of control samples which averaged 15.1 $\pm$ 0.7 units per 10^6 cells. The IC_{50} concentration of KCN was 1 mM.

Like catalase, glutathione peroxidase mediates breakdown of H_2O_2. Glutathione reductase restores glutathione to its reduced state after oxidation by glutathione peroxidase. Although these enzymes are important for cellular homeostasis under conditions of oxidative stress, no inhibition by cyanide of glutathione peroxidase in PC12 cells was detected, and glutathione reductase

activity was variable in the presence of cyanide. Therefore, these enzymes could not be used to screen for antidotal activity against cyanide.

Cytosolic-Free Calcium

The calcium-sensitive fluorescent probe, fura-2, was used to determine free intracellular calcium [Ca(i)] in the cells (Grynkiewicz et al., 1985). Fura-2 is a sensitive probe in detecting low levels of Ca(i) by measuring the spectrum at dual excitation 340 nm and 380 nm and a fixed emission of 510 nm. PC12 cells were loaded with 6 μM fura-2/AM (acetomethoxy ester) in loading buffer (RPMI with 20 mM HEPES and 5% fetal bovine serum) by incubating at 25°C for 45 min. The cell suspension was washed twice with incubation buffer, and cells were treated with the compounds to be screened for 15 min. Then cyanide (5 mM EC_{50}) was added and 5 min later changes in fluorescence were noted. The fluorescence of fura-2 was calibrated at the end of each assay. The fluorescence maximum (F_{max}) was measured by adding 20 μl of 10% Triton X-100, and the fluorescence minimum was determined by adding 20 μl of 5 mM EGTA in 0.1 M Tris base. The Ca(i) was calculated by the Spectra-Calc$^{\circledR}$ computer program (Hitachi Ltd., Tokyo, Japan) using the following formula:

$$Ca(i) = K_d(R-Rmin)/Rmax-R).(S_{f2}/S_{b2})$$

Where R = Fluorescence at 340 nm divided by fluorescence at 380 nm at a given time.

Rmax = ratio of fluorescence of 1 μM fura-2 at 340 nm/380 nm in a 1 mM Ca-EGTA buffer.

S_{f2} = fluorescence at 380 nm of 1 μM fura-2 in a 0 nm Ca-EGTA buffer.

S_{b2} = fluorescence at 380 nm of 1 μM fura-2 in a 1 mM Ca-EGTA buffer.

K_d = 224 nM.

Ca(i) was measured as nM and expressed as percent control.

Hydroperoxide Generation

The assay is based on conversion of 2,7-dichlorofluorescein to the fluorescent form of the same compound by hydroperoxides (LeBel et al., 1990, Mattia et al., 1991). PC12 cells were harvested and suspended in 10 ml of KRB solution, 10 μl of 20 mM dye was added and the mixture kept at room temperature for 15 min. Then the cells were washed two times with KRB solution by centrifugation at 5000 g for 8 sec. After centrifugation, cells were resuspended in KRB solution to give 4 x 10^6 cells/ml. Fluorescence was measured in a Hitachi Model F-2000 Spectrofluorometer before and for 5 min after addition of 1 mM KCN (excitation 475 nm, emission 525 nm). A standard curve was constructed using H_2O_2 and the data was analyzed using the Spectra-Calc® program. Measurements were recorded as μmol H_2O_2 generated/10^7 cells and were expressed as percent of KCN-induced peroxide level.

Although H_2O_2 is used as the standard for the fluorescent assay of peroxide, other hydroperoxides, e.g., lipid peroxides, are also detected by the assay.

Preliminary work in the screen involved estimation of cyanide-induced lipid peroxides using the conjugated diene method

(Johnson *et al.*, 1987a). The procedure is carried out under nitrogen since conjugated dienes form spontaneously in air. The conjugated dienes develop slowly from hydroperoxides generated previously by cyanide. Since this assay is laborious and variable and only a remote reflection of peroxide production, hydroperoxides were measured directly with 2,7-dichlorofluorescein.

Cytochrome Oxidase

Cytochrome oxidase activity was determined spectrophotometrically by measuring the rate of oxidation of reduced cytochrome c by cell homogenates after a 30 min exposure of the cells to cyanide at $37^{\circ}C$ (Isom and Way, 1984). The incubation mixture was placed in sealed cuvettes and the determinations were conducted at $25^{\circ}C$. The analytical procedure consisted of adding 2.4 ml of 30 mM Tris-HCl buffer (pH 7.4) to a 3 ml cuvette followed by 0.5 ml of reduced cytochrome c. The homogenate (0.1 ml) was added to the assay mixture and the decrease in absorbance at 550 nm was recorded over a 3 min period in a Perkin Elmer Lambda 3B Spectrophotometer. Potential antidotes were tested against an IC_{50} concentration of KCN (5 μM). Finally, 0.2 ml of saturated potassium ferricyanide was added to oxidize completely the cytochrome c, and the absorbance was redetermined to estimate maximal substrate oxidation. Cytochrome oxidase activity was noted as moles of cytochrome c oxidized/min/10^6 cells and was expressed as percent control.

Dopamine Release

Dopamine released by PC12 cells during 30 min incubation was estimated in the supernatant after centrifugation of the cells (5000 g for 8 sec). Analyses were conducted using high performance liquid chromatography (HPLC) with electrochemical detection (Saller and Salama, 1984). The amines were separated on a C-18 reversed phase column (3 mm X 100 mm) with an isocratic flow rate of 1 ml/min. An LC 4B amperometric detector with glassy carbon electrode set at 0.65V versus an Ag/AgCl reference electrode (Bioanalytical Systems Inc., West Lafayette, IN) was used to detect the catecholamines. Chromatograms were evaluated using a 427A integrator (Beckman, San Ramon, CA). The mobile phase consisted of monochloroacetic acid, 0.15 M; sodium octyl sulfonate, 0.13 mM; disodium EDTA, 0.67 mM; sodium hydroxide 0.12 M and 1.5% acetonitrile; pH was adjusted to 3.1 with phosphoric acid. The system was calibrated with dopamine and catecholamine standards and 3,4-dihydroxybenzylamine was used as an internal standard. The main catecholamine released from PC12 cells by cyanide was dopamine. Only small amounts of norepinephrine were released and no epinephrine was detected. Dopamine secretion in response to 1 mM KCN was measured as $ng/10^6$ cells and the results expressed as percent above basal secretion. In some instances, it was necessary to use a concentration of KCN higher than 1 mM (e.g., 5 mM) to obtain an adequate dopamine release.

Drug Combinations

In an effort to maximize protection against cyanide in vitro, compounds which were effective when used alone were

combined with one another and employed to block the actions of cyanide. Substances were selected to compliment one another; for example, a compound effective against cyanide-induced dopamine release (allopurinol) was combined with pyruvate, an antioxidant. In some instances three compounds were employed simultaneously in an attempt to achieve a high level of protection against cyanide in vitro. All antidotes were employed at a concentration of 10 μM except for combination studies involving sodium thiosulfate, in which 100 μM was used. Sodium thiosulfate was used in a high concentration in vitro in the combination studies since it is also employed at a high dose (1 g/kg) in vivo in mice as a cyanide antidote.

LD$_{50}$ Determinations

To test the validity of the in vitro screen, compounds active against cyanide toxicity in PC12 cells were examined for protection against lethal doses of cyanide in mice. Non-Swiss Albino mice (21-26 g, Harlan Sprague-Dawley, Indianapolis, IN) were given standard laboratory chow and water ad libitum. They were maintained in a temperature/humidity controlled environment with a light-dark cycle 8 am to 8 pm. Potential antidotes were administered prior to sc doses of cyanide. Sodium thiosulfate (1 g/kg ip 15 min before cyanide) was also administered along with the other substances since many cyanide antidotes are active only in the presence of thiosulfate. LD$_{50}$ values were calculated according to a commercially available computer program (Chou and Chou, 1985). The "potency ratio" was calculated for each compound by dividing the LD$_{50}$ of cyanide alone into the LD$_{50}$ of

cyanide in mice given the antidote plus thiosulfate. These values were then plotted (Figure 6) against _in_ _vitro_ test scores given in Table 1.

RESULTS

Antioxidant Defense Enzymes

Catalase and superoxide dismutase (SOD, CuZn form) are very susceptible to the action of cyanide (Solomonson, 1981). Only one of the 39 compounds screened _in_ _vitro_ prevented SOD inhibition by cyanide (trifluoperazine, Table 1) and only a few substances, including carbamazepine had significant ($p<0.05$) ability (18-28%) to protect catalase against cyanide (Figure 1, Table 1).

Cytosolic-Free Calcium

Several of the potential antidotes screened limited the rise in intracellular calcium caused by cyanide. Retinol acetate, nifedipine, mannitol and naloxone were most effective, preventing between 38% and 47% of the cyanide-induced increase in cytosolic-free calcium (Figure 2, Table 1).

Peroxide Formation

Some prevention of hydroperoxide generation by cyanide was detected with one third of the compounds tested. Pyruvate and mercaptopyruvate were nearly 100% effective, whereas imipramine, bretylium and carbamazepine blocked between 61 and 71% of the effect (Figure 3, Table 1). Alpha-ketoglutarate also decreased the amount of peroxides formed by over 50% (Figure 3, Table 1).

Table 1. Relative Effectiveness of Potential Antidotes (0.01 mM) Against Biochemical Effects of Cyanide

Compound	N=4 Inhib. Dopamine Release	N=4 Cytochrome Oxidase Inhibition	N=3 SOD Inhib.	N=4-5 Catalase Inhib.	N=2-5 Peroxide Generation	N=2-4 Cytosolic Calcium	Total Score
Carbamazepine	9.2±0.5*	0	0	1.4±0.6*	7.1±1.5*	3.3±0.3*	21.0
Phenytoin	10.0±0.8*	0	0	0.9±0.5	0	1.8±0.2*	12.7
Mannitol	5.1±1.1*	0.8±1.2	0	0.4±0.4	2.9±0.6	3.3±0.3*	12.5
Pyruvate	0	0	0	0.9±0.4*	9.2±0.6*	2.2±0.3*	12.3
Mercapto-pyruvate	0	1.8±0.7*	0	0.4±0.4	9.5±0.1*	0	11.7
Allopurinol	10.0±0.0*	0	0	0.7±0.6	0	0	10.7
α-keto-glutarate	2.3±1.3	0	0	0.9±0.5	5.6±1.5	1.7±1.3	10.5
Flunarizine	6.4±0.9*	0.7±0.5	0	0.3±0.3	0	3.0±0.5*	10.4
Imipramine	0	0.7±0.6	0	0.5±0.4	6.1±2.1	2.7±0.4*	10.0
Retinol Acetate	2.2±1.2	0	0	0.4±0.5	1.3±0.8	4.6±0.4*	8.5
Glyceralde-hyde	5.3±1.0*	0	0	0.4±0.2	2.6±1.2	0	8.3
Acetazolamide	2.0±1.2	0.5±0.7	0	0	3.7±0.4*	1.4±0.7	7.6
Trifluo-perazine	5.8±1.0*	0	1.1±0.2*	0	0	0	6.9
Nifedipine	3.2±1.1	0	0	0	0	3.6±0.3*	6.8
Tolazoline	5.0±1.2	0	0	0.1±0.2	0	1.6±0.2	6.7
Lidocaine	3.8±0.6*	0	0	0	2.0±1.5	0.9±0.9	6.7
Bretylium	0	0	0	0	6.3±0.4*	0	6.3
Hydrocortisone	3.1±0.7*	0	0	0	0	3.1±0.2*	6.2
Primidone	5.7±0.3*	0	0	0	0	0	5.7
Naloxone	0	0	0	0.5±0.6	0	4.4±0.6*	4.9
Phenoxy-benzamine	0	0	0	0	2.9±1.1	1.8±0.7	4.7
Hydroxytoluene	2.5±1.6	0	0	0	0	2.0±1.6	4.5
α-tocopherol	0	1.2±0.9	0	0.9±0.4*	0.6±2.0	1.4±1.1	4.1
NaNO$_2$	1.1±1.4	0.7±0.7	0	0	0	1.2±1.0	3.0
Na$_2$S$_2$O$_3$	0	1.8±0.6	0	0.4±0.3	0.7±0.3	0	2.9
Uric acid	0	0	0	0	1.7±0.8	0.9±1.4	2.6
Valproate	0	0.4±1.1	0	0	0	1.8±1.0	2.2
Glutathione	0	0	0	0.7±0.5	0	1.5±0.3	2.2

Centrophenoxine	0	0.7±1.3	0	0.4±0.3	1.0±1.7	0	2.1
Promethazine	0	0	0	0	0	1.7±1.7	1.7
Fructose	1.2±1.8	0	0	0.5±0.5	0	0	1.7
Lidoflazine	0	0	0	0	0	1.4±1.6	1.4
Ethosuximide	0	0	0	0	0	0.7±0.9	0.7
Chlorpromazine	0	0.6±0.6	0	0	0	0	0.6
Pentobarbital	0	0	0	0.1±0.0	0	0	0.1
Lazaroid	0	0	0	0	0	0	0
Clonidine	0	0	0	0	0	0	0
Phenacemide	0	0	0	0	0	0	0
Trimethadione	0	0	0	0	0	0	0

Ranking Scale = Values represent degree of blockade of the effects of cyanide. Complete reversal is assigned a value of 10, 80% reversal a value of 8, etc. The two antioxidant defense enzymes were grouped together and given a total value of 10 so that complete protection against the inhibitory effect of cyanide in any one enzyme system was assigned a value of 5. Asterisk indicates a significant effect at least at the 5% level compared to cyanide control.
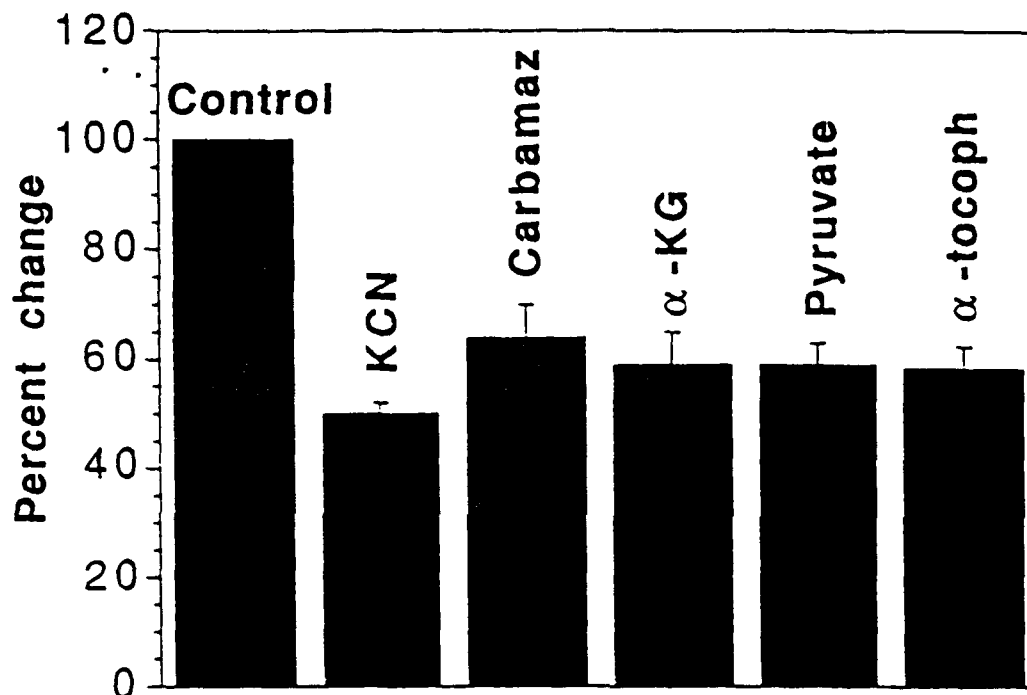
Figure 1. **Prevention of Cyanide-Induced Inhibition of Catalase by Potential Antidotes.** Antidotes (10 μM) were added to suspensions of PC12 cells 15 min prior to 55 μM KCN. Thirty min later, aliquots of homogenates were taken for assay of catalase activity. The effects shown are significant at the P < 0.05 level. Means $\pm$ SE are given for 4-5 observations. The first order rate constant (K) for catalase activity in control samples averaged 4.3 $\pm$ 0.4 x 10^{-4}. Carbamaz = carbamazepine, α-Tocoph = alpha tocopherol.
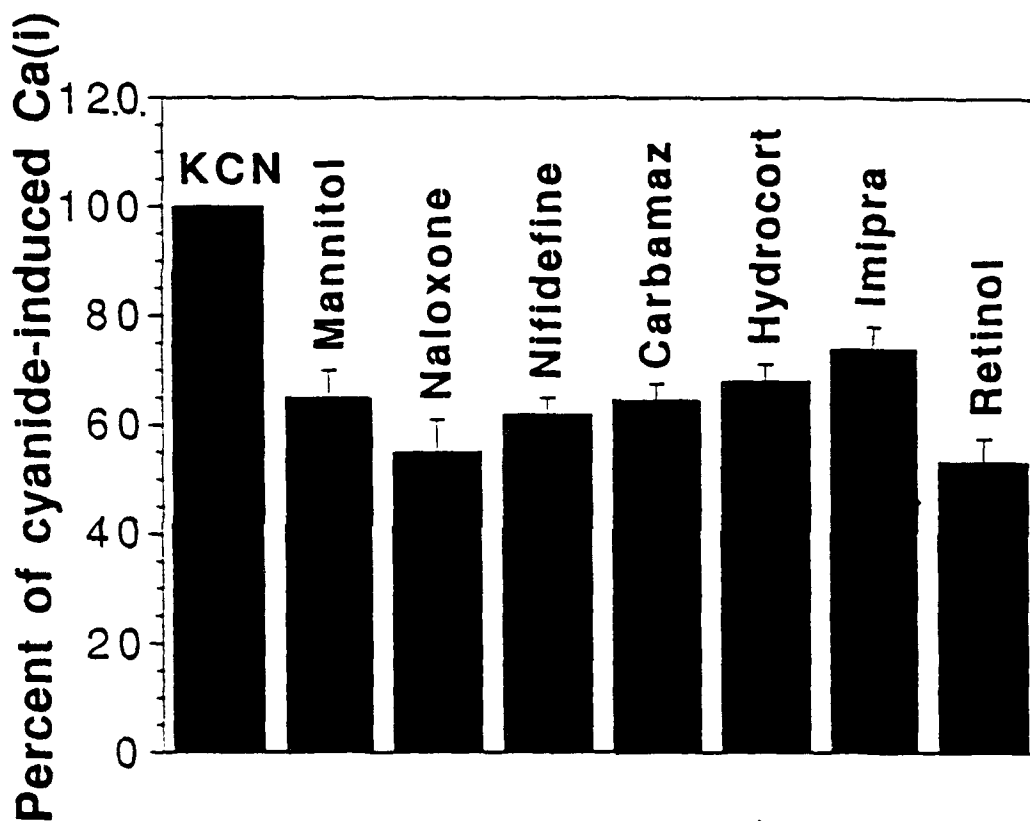
Figure 2. **Prevention of Cyanide-Induced Increases in Cytosolic Calcium by Potential Antidotes.** PC12 cells were pretreated with the antidotes (10 μM) for 15 min prior to addition of 5 to 10 mM KCN. Changes in cellular free calcium were estimated fluorimetrically. The effect of each of the above compounds was significant at least at $p < 0.05$. Means $\pm$ SE are shown for 3 to 13 observations. Basal calcium concentration in control cells was 49.2 $\pm$ 1.8 nM. Hydrocort = hydrocortisone and Imipra = imipramine
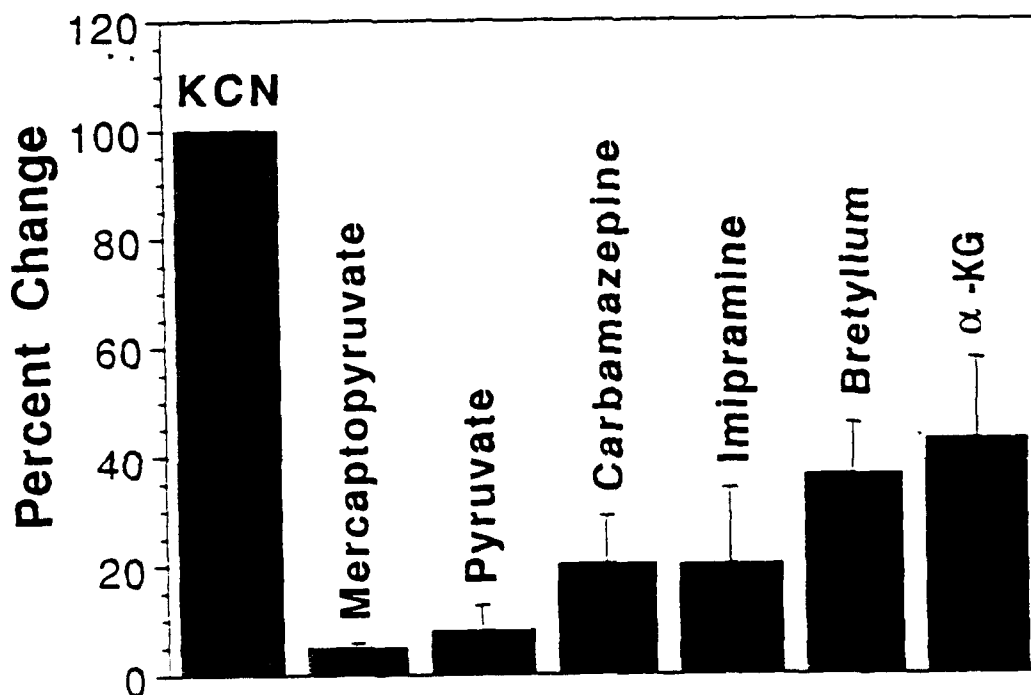
Figure 3. Inhibition of Cyanide-Induced Increases in Hydroperoxides. PC12 cells were pretreated with the antidotes (10 μM) for 15 min prior to addition of KCN. Hydroperoxides were measured fluorometrically 5 min after cyanide addition. Generation of hydroperoxides in cyanide treated cells was 1.98 $\pm$ 0.43 μmol $H_2O_2/10^6$ cells per 5 min. N = 2 to 8. α-KG = alpha-ketoglutarate.

Cytochrome Oxidase

Cyanide strongly inhibits cytochrome oxidase, so it is not surprising that none of the compounds tested completely protected against cyanide inhibition of this enzyme. However, partial protection (18%) was provided by mercaptopyruvate, and several compounds had lesser protective effects (Figure 4, Table 1).

Dopamine Release

Phenytoin and allopurinol completely inhibited dopamine release from PC12 cells by cyanide; and carbamazepine inhibited 92% of the cyanide-induced secretion (Figure 5, Table 1). Another anticonvulsant, primidone, inhibited the response by 57%. The calcium channel blockers, flunarizine and nifedipine also showed appreciable inhibitory actions (64 and 32% respectively) along with glyceraldehyde (53%) and trifluoperazine (58%).

Testing of Combinations of Antidotes In Vitro

In an effort to maximize protection against cyanide in vitro, compounds which were effective when used alone were combined with one another and employed to block the actions of cyanide. After completing the whole range of six biochemical assays in vitro using various combinations (Table 2) it is evident that the ranking scores are lower for many combinations than for the antidotes used separately. For example, carbamazepine alone gave a ranking score of 21.0 but when combined with pyruvate (which had a ranking score of 12.3), the resulting score was only 7.2. It appears that antagonism occurs in vitro when certain antidotes are combined in an attempt to block cyanide's biochemical actions.
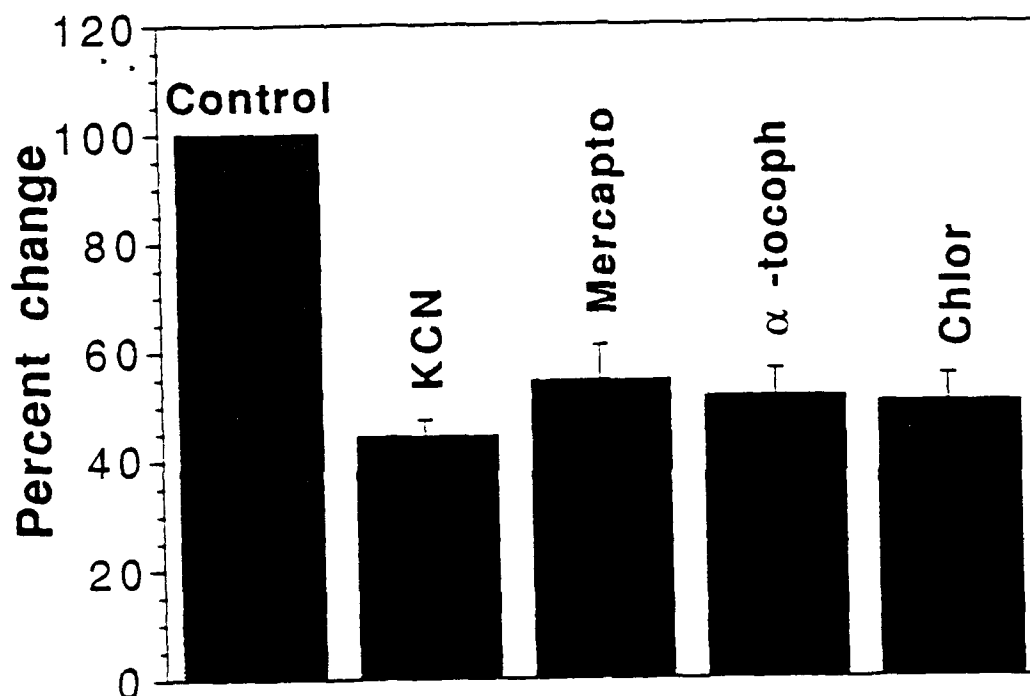
Figure 4. Protection of Cytochrome Oxidase Against Inhibition by Cyanide. Potential antidotes (10 μM) were added to suspensions of PC12 cells 15 min prior to cyanide. After 30 min incubation, aliquots of cell homogenate were taken for assay of cytochrome oxidase. The effect of mercaptopyruvate was significant at the 5% level. Means $\pm$ SE are shown for 4-5 observations. The first order rate constant (K) for cytochrome oxidase activity in control samples was 2.9 $\pm$ 0.5 x 10^{-2}. Mercapto = mercaptopyruvate, α-tocoph = alpha tocopherol, Chlor = chlorpromazine.
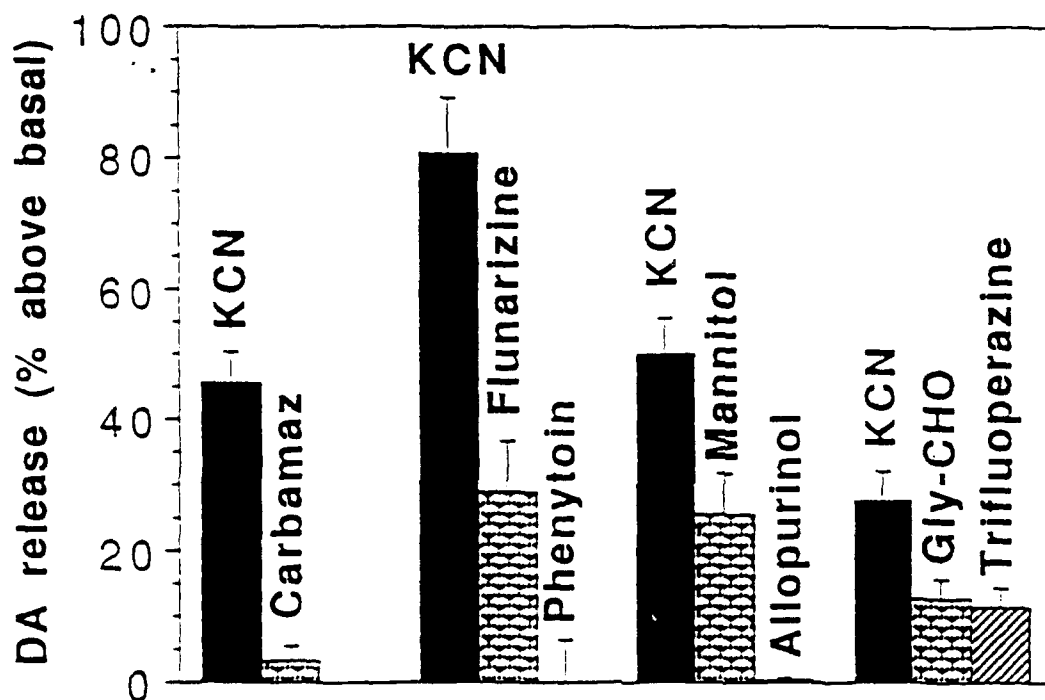
Figure 5. **Prevention of Cyanide-Induced Dopamine Release by Potential Antidotes.** Antidotes (10 μM) were added to the suspension of PC12 cells 15 min prior to 1 mM cyanide. Thirty min later, cells were pelleted by centrifugation and aliquots of the supernatant taken for analysis of dopamine by HPLC-EC. Control responses to KCN are shown along with responses in the presence of antidotes. Means $\pm$ SE are shown for 4-5 determinations. Dopamine release from control untreated samples was 77.0 $\pm$ 11.5 ng/10^6 cells. Carbamaz = carbamazepine, Gly-CHO = glyceraldehyde.

Table 2. Summary of Effectiveness of Combinations of Cyanide Antidotes In Vitro

Combo	Inhib. Dopamine Release	Cytochrome Oxidase Inhibition	SOD Inhib.	Catalase Inhib.	Peroxide Generation	Cytosolic Calcium	Total Score
Thiosulf + Carb	5.9±3.4*	1.4±0.1	0.9±0.1	0.3±0.1	8.3±1.7*	5.0±3.2	21.8
Thiosulf + Mercap + Carb	6.6±2.3*	2.4±1.8	0.4±0.6	0.6±0.1	9.0±1.2*·	0	19.0
Pyruv-Nal-Flu	6.4±1.9	0	0.9±0.3	0	1.2±0.2*	3.0±1.5	11.5
Pyruv-Nal	2.4±1.7	2.8±1.4	0	0	1.2±0.5	2.9±0.5*	9.3
Pyruv-Nal-Allo	4.1±2.4	0	0	0	1.6±0.3	2.6±1.1*	8.3
Pyruv-Flu	5.9±2.0	0	0	0	0	1.9±1.0	7.8
Carb-Pyruv	2.9±0.7	1.3±1.1	0	0.6±0.1	2.4±0.3*	0	7.2
Carb-Nal	2.9±1.4	0.5±0.4	0	0.4±0.3	1.7±0.6	0	5.5
Thiosulf	0	3.4±0.4*	0	0.4±0.0	0	1.5±2.1	5.3
100 μM alone							
Pyruv-Allo	2.4±1.9	0	0	0	0	1.9±0.2*	4.3
Carb-Mann	3.8±1.4	0	0	0	0	0	3.8
Carb-α Keto	3.3±1.8	0	0	0	0	0	3.3
Thiosulf + Mercap	0	0	0	0	2.4±0.8	0	2.4
Allo-Imi	0	0	0	0	0	2.2±0.7	2.2
Flu-Imi	0	0	0	0	0	1.9±0.4	1.9
Mann-α Keto	0	0	0	0	0	0	0
Mann-Nal	0	0	0	0	0	0	0
Mann-Pyruv	0	0	0	0	0	0	0
Mann-Imi	0	0	0	0	0	0	0

NOTE: A minimum of four determinations was made for each value shown. All antidotes were used at a 10 μM concentration except for sodium thiosulfate which was used at a 100 μM level. Asterisk indicates a significant effect at least at the 5% level compared to cyanide control.

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However, the combination of carbamazepine and thiosulfate was as effective as carbamazepine alone in blocking the biochemical effects of cyanide. Accordingly this combination was used _in_ _vivo_ and was included in Figure 6 for comparison of antidotal activity with _in_ _vitro_ scores.

Correlation with In Vivo Results

A plot of the protection against cyanide _in_ _vivo_ (LD_{50} values in the presence of sodium thiosulfate) and the test scores in the _in_ _vitro_ screen is illustrated in Figure 6. The _in_ _vivo_ protection score (LD_{50}) was obtained from the literature for chlorpromazine (Way and Burrows, 1976) in combination with sodium thiosulfate. The value for sodium thiosulfate alone (Moore et al., 1986) was also obtained from the literature. Other LD_{50} values were determined in our lab. The correlation coefficient of the curve was 0.876 (p<0.05).

DISCUSSION

Present studies illustrate that a well-characterized neuron-like cell line can be used to assess a series of biochemical and neurochemical markers of toxicity. Blockade of the biochemical effects of cyanide at the cellular level is then used as the basis of the rational selection of effective cyanide antidotes. Two findings support the validity of this approach. First, several known biochemical antidotes (e.g., naloxone, pyruvate, mercaptopyruvate and flunarizine) scored relatively high in the screen, and secondly, effectiveness in the screen can be correlated with ability of selected compounds to prevent death due to cyanide in mice. Known cyanide antidotes which have
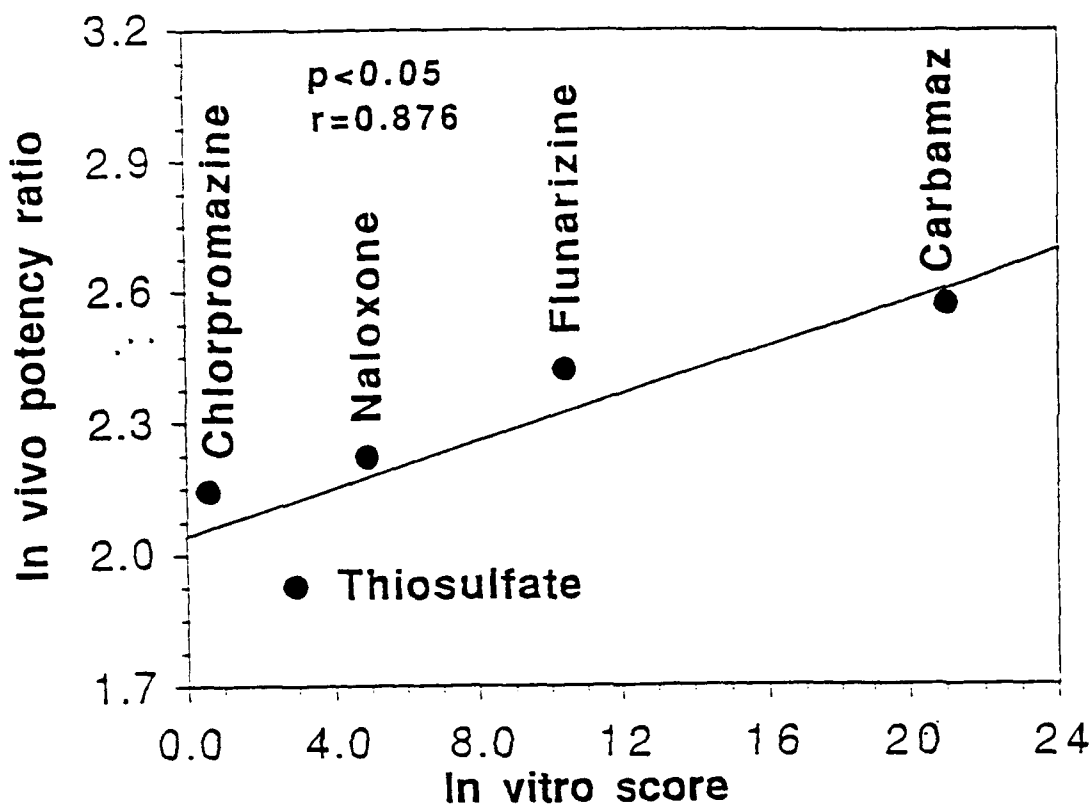
Figure 6. Correlation Between In Vitro Test Scores and In Vivo Actions of Cyanide Antidotes. In vitro test scores were taken from Table 1 and correlated with in vivo potency. The potency ratio is the LD_{50} of cyanide in mice divided into the LD_{50} of cyanide in mice pretreated with thiosulfate (1 g/kg, ip, 15 min prior to KCN) and the antidote shown. Doses, routes and time intervals were as follows: chlorpromazine HCl 10 mg/kg, sc, 30 min prior to cyanide (data taken from Way and Burrows, 1976); flunarizine 100 mg/kg, ip, 1 hr before cyanide; naloxone HCl 20 mg/kg, ip, 10 min prior to cyanide; carbamazepine 50 mg/kg, ip, 1 hr before cyanide; sodium thiosulfate alone was given 15 min before cyanide (data taken from Moore et al., 1986). The correlation is significant at P < 0.05.

mechanisms not operative in vitro (e.g., nitrate and thiosulfate) scored low in the screen, as anticipated, since the cells do not generate methemoglobin and have a low level of rhodanese for conversion of cyanide into thiocyanate. Thus the screen appears to be capable of identifying biochemical cyanide antidotes, but would not be expected to detect sulfur donors or methemoglobin formers. .

Blockade of mitochondrial cytochrome oxidase with depletion of cellular energy stores has long been considered the primary biochemical effect of cyanide. However, recent reports showing an equivalent degree of cytochrome oxidase inhibition in brains and hearts of mice which died and those that survived doses of cyanide, cast doubt on this concept (Petterson and Cohen (1985, 1986). Additionally, Yamamoto (1989) showed that mice rendered unconscious by cyanide had no decrease in brain ATP whereas liver ATP decreased to 60% of control. Thus cytochrome oxidase inhibition may explain only part of the toxic syndrome caused by cyanide, and for this reason other biochemical actions of cyanide were also considered in the screen for antidotal activity.

Cyanide's effect on neural systems can be very rapid in onset. Aitken and Braitman (1989), using transverse slices of guinea pig hippocampus, found that application of cyanide (10 to 200 μM) almost immediately depressed synaptic transmission between Schaffer collateral commissural fibers and CA1 pyramidal cells. Direct electrical excitability of axons was not affected since 500 μM cyanide had no effect on antidromic activation of pyramidal cells. Cyanide's effect reversed rapidly on washout,

suggesting that cyanide has a direct action on neurons not mediated by metabolic inhibition.

Not all neural systems are inhibited by cyanide. Persson et al. (1985) and Cassel and Persson (1992) found rapid release of brain neurotransmitters after ip injection of NaCN. Dopamine, for example, decreased in striatum within 60 sec after cyanide administration. A related study showed that cyanide released [³H] norepinephrine from rat brain cortical slices and also released dopamine from PC12 cells (Kanthasamy et al., 1991b). We have also demonstrated that catecholamine blood levels increase on administration of cyanide to mice (Kanthasamy et al., 1991a). Furthermore, glutamate secretion from mouse cortical, hippocampal and cerebellar slices was induced by cyanide partly in a calcium-dependent manner (Patel et al., 1991). Whether cyanide-induced neurotransmitter release is due to metabolic inhibition is not known. However, many symptoms of cyanide intoxication, e.g., tremor and convulsions, may be due to neurotransmitter release. Thus a measure of the ability of a potential antidote to inhibit cyanide-induced neurotransmitter release was included as part of the screen for cyanide antidotes in the present study.

Another important biochemical change after cyanide exposure involves calcium. Cyanide-induced tremors in mice parallel an increase in whole brain calcium (Johnson et al., 1986). Both tremors and accumulation of brain calcium are diminished by pretreatment with diltiazem, a calcium channel blocker (Johnson et al., 1986). Similar cyanide-induced increases in calcium have been reported in rat pheochromocytoma (PC12) cells (Johnson et

al., 1987a). Recent studies indicate that cyanide-induced glutamate release may be involved in elevating $Ca_{(i)}$ (Patel *et al.*, 1991). Elevated cytosolic calcium following cyanide exposure may generate hydroperoxide radicals which lead to lipid peroxidation and cell damage (Johnson *et al.*, 1987b). Diltiazem pretreatment blocks membrane lipid peroxidation in mouse brain caused by cyanide (Johnson *et al.*, 1987b). A complete biochemical screen for cyanide antidotal activity should include measures of the ability of potential antidotes to block cyanide-induced elevation of cell calcium and hydroperoxide generation.

If oxygen radicals mediate cell damage after cyanide exposure, then antioxidant systems in cells would be critical for protection against cyanide. This is especially important since enzymes which degrade oxygen radicals are inhibited by cyanide (Ardelt *et al.*, 1989). Effectiveness of potential antidotes in protecting antioxidant defense enzymes against cyanide may be important clinically and should be evaluated in any screen which attempts to identify cyanide antidotes.

The data provide insights into mechanisms of antidotal activity. For example, cyanide intoxication in mice is antagonized by naloxone (Leung *et al.*, 1986). Naloxone also blocks the rise in cytosolic free calcium induced by cyanide in the present study. It is possible that the antidotal effect of naloxone is not limited to antagonism of opiate receptors but may also involve an effect on cellular calcium handling process.

The anticonvulsants, phenytoin and carbamazepine, were both effective in blocking cyanide-induced dopamine release. Of all

the 39 compounds tested, only allopurinol (which was recently reported to have anticonvulsant effects [DeMarco and Zagnoni, 1988]) equalled the potency of the anticonvulsants. The main anticonvulsant action of phenytoin and carbamazepine is thought to be blockade of sodium channels to inhibit high frequency discharges around epileptic foci (Willow et al., 1985). Minimal disruption of normal neuronal traffic is produced by these drugs. These results suggest that cyanide may enhance sodium influx through channels responsible for high frequency discharges. In support, epileptiform discharges have been observed in guinea pig hippocampal slices after cyanide exposure in vitro (Lebeda et al., 1990).

Recently Peruche and Krieglstein (1991) reported on the use of neuroblastoma cells to test for substances which protect against 1 mM sodium cyanide. The cells were exposed for 6 hr to cyanide in a glucose-free medium but protective compounds were present 30 min prior to addition of cyanide and for 24 hr after cyanide removal. Two parameters were measured 7 days after removal of the potential antidotes; ATP levels and protein content of the material in the incubation flask. The latter value presumably reflected lack of cell growth after cyanide exposure. Three compounds were tested by Peruche and Krieglstein and in the present study. Chlorpromazine and flunarizine exhibited some weak protective effects in both studies. Phenytoin failed to protect neuroblastoma cells but scored relatively high in the PC12 screen. The main action of phenytoin in PC12 cells was to block dopamine release by cyanide. This

action may be inconsequential to the survival and growth of cyanide-treated cells in culture, but may be important in vivo where cyanide induces the release of large amounts of neuromediators (Kanthasamy et al., 1991a, 1991b).

Ray et al. (1991) also reported the use of neuroblastoma (NG 108-15) cells as a model for studying cyanide and its antidotes. The cells were sensitized to cyanide by blockade of anaerobic metabolism with iodoacetate or with 2-deoxyglucose. In the presence of 2-deoxyglucose, 0.01 mM cyanide reduced ATP levels by 75% in 1 min, and this effect was prevented in a dose-dependent manner by cyanide scavengers. It remains to be demonstrated whether biochemical cyanide antidotes are effective in NG 108-15 cells treated with 2-deoxyglucose and cyanide. Furthermore, it will be interesting to determine to what extent substances which block the decrease in ATP by cyanide in neuroblastoma cells prevent the toxic consequences of cyanide.

Cyanide intoxication appears to be a complex syndrome, and many factors contribute to the total cellular insult. Substances which counteract the biochemical alterations induced by cyanide may be useful in supplementing the standard treatments involving a scavenger and a sulfur donor. This study demonstrates that a series of biochemical neurochemical assays in a neuron-like cell line can be used as the basis of an in vitro toxicological screen and can be used to predict the efficacy of potential biochemical cyanide antidotes.

CONCLUSIONS

1. The following biochemical alterations are produced by cyanide in rat pheochromocytoma (PC12) cells.

    a) increased cell calcium

    b) dopamine release

    c) hydroperoxide generation

    d) enzyme inhibition

        i) cytochrome oxidase

       ii) catalase

      iii) superoxide dismutase

2. Many of these cyanide-induced changes can be attenuated by pretreatment of the cells with drugs.

3. The most effective drugs in each of the biochemical assays were as follows:

    a) increased cell calcium: retinol acetate, naloxone

    b) dopamine release: phenytoin, allopurinol, carbamazepine

    c) hydroperoxide generation: pyruvate, mercaptopyruvate

    d) enzyme inhibition:

        i) cytochrome oxidase, mercaptopyruvate

       ii) catalase: carbamazepine

      iii) superoxide dismutase: trifluoperazine

4. Generally, combinations of drugs offered no advantage over drugs used alone to block the biochemical effects of cyanide.

5. Many substances which are effective against cyanide-induced biochemical changes in PC12 cells are also effective in preventing death induced by cyanide in mice.

6. A significant correlation exists between abilities of select drugs to block biochemical alterations by cyanide in PC12 cells and their abilities to prevent cyanide lethality in mice.

7. Results of the _in vitro_ screen suggest that carbamazepine, allopurinol and mannitol may be of therapeutic value against cyanide toxicity. These substances and their analogs may be worthy of further study as cyanide antidotes.

8. Preliminary studies show that addition of rhodanese along with thiosulfate enhances _in vitro_ effectiveness (Figure 7). Thus, biochemical antidotes added along with rhodanese and thiosulfate have enhanced antidotal activity. Addition of rhodanese and thiosulfate may make the system more parallel to the _in vivo_ situation and may provide a more reliable _in vitro_ indication of antidotal effectiveness against cyanide.
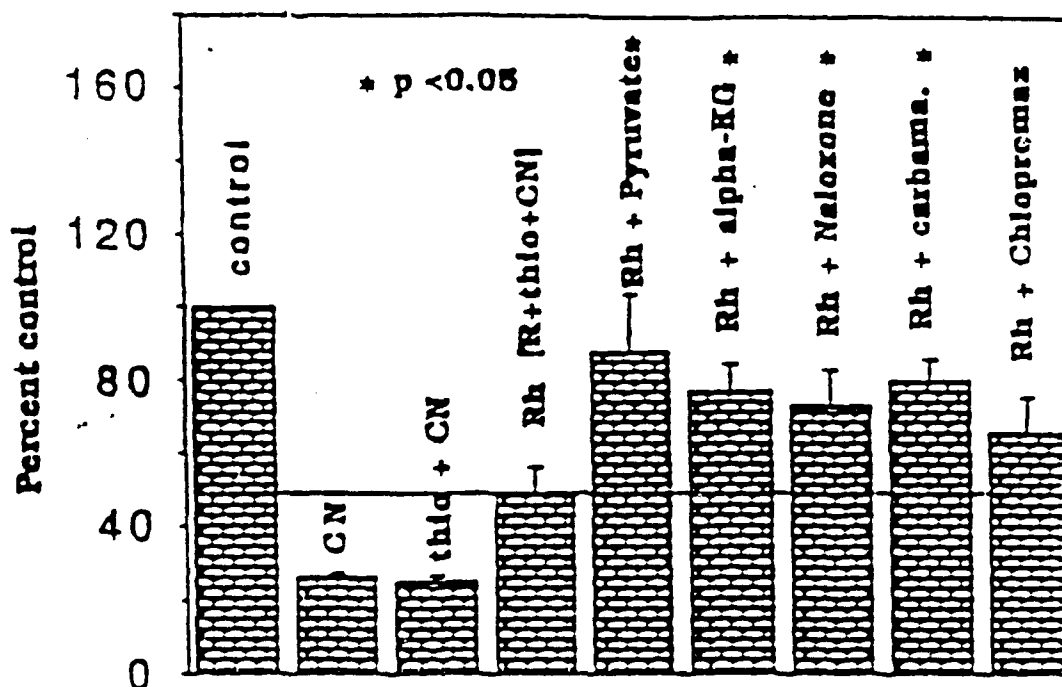
Figure 7. Effect of KCN (100 μM) on Cytochrome Oxidase Activity in PC12 Cells Preincubated with Various Compounds. Cells were preincubated 15 min before the addition of KCN with rhodanese (2.5 units), sodium thiosulfate (100 μM) and the specific test compound (10 μM). At zero time, KCN (100 μM) was added and the cells incubated for 30 min, the incubation was terminated and cytochrome oxidase activity determined. Cytochrome oxidase activity is expressed as percent of control cells (no treatment). Each value represents the mean ± SEM of 3-6 separate studies and the asterisks indicate significant difference (P<0.05) from cells treated with rhodanese + thiosulfate (Rh group).

    Key: CN: KCN treatment
           Thio: Sodium thiosulfate treatment
           Rh: Rhodanese + thiosulfate treatment
           Rh + compound: Rhodanese + thiosulfate + compound
                 treatment
Pyruvate = sodium pyruvate; alpha-KG = alpha ketoglutarate; naloxone = naloxone HCl; carbama. = carbamazepine; chlorpromz. = chlorpromazine.

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APPENDIX I

Abbreviations:

Pyruv	=	pyruvate
Nal	=	naloxone
Allo	=	allopurinol
Flu	=	flunarizine
Imi, imipra	=	imipramine
Carb, carbamaz	=	carbamazepine
α Keto, α-KG	=	α ketoglutarate
Mann	=	mannitol
Thiosulf	=	thiosulfate
Mercap, mercapto	=	mercaptopyruvate
α-tocoph	=	α tocopherol
chlor	=	chlorpromazine
hydrocort.	=	hydrocortisone
Gly-CHO	=	glyceraldehyde

NOTE on the Final Data:

Since experiments were repeated to increase the number of observations, the values given in the final report may differ from those given in the quarterly or midterm reports.

Appendix II

Publications resulting from the contract support:

1. Development of an <u>in</u> <u>vitro</u> Screen for Biochemical Cyanide Antidotes. Joseph L. Borowitz, Anumantha G. Kanthasamy and Gary E. Isom. Proceedings of the 1991 Medical Defense Bioscience Review, U.S. Army Research and Development Command, pp. 269-272.

2. Use of PC12 Cells as an <u>in</u> <u>vitro</u> Screen for Biochemical Cyanide Antidotes. Borowitz, J.L., Kanthasamy, A.G. and Isom, G.E. 6th International Symposium on Chromaffin Cell Biology. Marburg, F.R. Germany. Abstract presented Aug. 18-23, 1991.

3. Anticonvulsants Block Cyanide-induced Dopamine Release from PC12 Cells. A. Kanthasamy, J.L. Borowitz and G.E. Isom. In preparation.

4. Use of PC12 Cells as a Neurotoxicological Screen: Characterization of Anticyanide Compounds. A. Kanthasamy, J.L. Borowitz and G.E. Isom. In preparation.

Personnel receiving pay from the contract:

Gary E. Isom, Ph.D., Principal Investigator
Joseph L. Borowitz, Ph.D., Co-investigator
Anumantha G. Kanthasamy, Ph.D., Postdoctoral Fellow
Lia Huang, former Technician
Pamela Mitchell, Technician

Note: No graduate students were supported financially, but training of several students and postdoctoral fellows was enhanced as a result of experiments conducted under this contract.