Commentary

A RATIONALE FOR THE DESIGN OF CELL-SPECIFIC TOXIC AGENTS: THE MECHANISM OF ACTION OF 6-HYDROXYDOPAMINE

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Received 1 November 1976

Progress towards the development of new therapeutic agents is increasingly dependent upon rational experimental designs patterned after the mechanism of action of 'lead' discoveries [1]. An elusive but much sought-after goal of medical research has been the development of specific, site-directed therapeutic agents. These agents would have little or no general toxicity towards the host but would seek-out a specific pathogen or cell type and then express a lethal toxicity. The rationale presented in this commentary is patterned after the mechanism of action of a specific, site-directed toxic agent. The behavior of certain derivatives of biogenic amines, of which 6-hydroxydopamine (6-HD) is the prototype, displays an impressive cellular specificity coupled with a lethal toxicity which is expressed only at specific cell types [2–4]. This phenomenon occurs as a consequence of the substrate specificity of an active transport mechanism located in the plasma membrane of sympathetic neuronal terminals. This mechanism accepts the amine derivative in place of the naturally occurring amine and thus raises the intracellular concentration of the derivative to a critical concentration which triggers a local toxicity. Thus, in this case there are two requirements for the expression of specific site-directed toxicity. One, the presence of an active transport mechanism localized in the plasma membrane of the cell and two, the molecular properties of the derivative which is transported into the cell. The present commentary is restricted to a description of the mechanism by which amine derivatives, such as 6-HD, produce a localized neurodegeneration of sympathetic nerve terminals. However, specific transport systems are known which can elevate the intracellular concentration of a variety of substances including amino acids, sugars, fats, and fatty acids. Such transport systems are present presumably to differing degrees in many, if not all, cell types including neoplastic cells. The present approach to the design of cell-specific toxic agents does not appear to be generally applicable to transport system common to many cell types. However, the variation in specificity and affinity for various compounds among different cell-types is sufficient to justify the proposed rationale. If such compounds also possessed the molecular properties common to the active form of 6-HD it is possible that they might be cell-specific toxic agents. An example of the application of this rationale has already led to the development of 5,6-dihydroxytryptamine [5] and 5,7-dihydroxytryptamine [6] – compounds which appear to induce degeneration in serotonergic neurons. Thus, the critical question pertaining to this rationale for the development of site-specific toxic agents concerns specific molecular mechanism by which 6-HD precipitates the cytotoxicity.

At present two mechanism for the specific cytotoxic action of 6-HD in catecholamine-containing neurons have been proposed; one mechanism invokes the generation of cytotoxic moieties such as hydrogen peroxide, superoxide radical and hydroxide radical during the intraneuronal autoxidation of 6-HD [7] and the other invokes covalent reaction of the primary reaction product, the 1,4-paraquinone of 6-HD (6-HDQ) with sulfhydryl groups on neuronal

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proteins resulting in an extensive cross-linking and inactivation of protein constituents essential for the survival of the nerve terminal [8,9].

It is now clear that the cellular specificity of the cytotoxic action of 6-HD is a consequence of the specificity of the uptake mechanism for amines located in the plasma membrane of the neuronal terminal. Inhibition of this 'membrane pump' blocks the entrance of 6-HD into the axoplasm of the neuron and thus prevents the cytotoxic effect. However, for the cytotoxicity to be manifest a critical intraneuronal concentration of 6-HD must be achieved through the action of the transport system. While 6-HD has been shown to interact with specific constituents within the neuron such as the amine storage vesicle, monoamine oxidase, dopamine-B-oxidase and tyrosine hydroxylase, there is no evidence that these interactions are in any way specifically related to the cytotoxic effect [10]. On the contrary, both of the proposed mechanisms suggest that the rapid generation within the nerve terminal of highly reactive substances derived from the autoxidation of 6-HD elicits a nonspecific inactivation of cellular constituents.

Studies of the interaction of 6-HD with various model proteins in vitro have clearly demonstrated that 6-HD reacts with nucleophilic groups on proteins to form a stable covalent bond. [11,9]. This reaction occurs readily at physiological pH and has an absolute requirement for molecular oxygen. The maximum extent of reaction of 6-HD with various proteins was achieved following unfolding of the tertiary structure of the protein in 4 M urea thus exposing sterically protected nucleophilic sites to attack presumably by 6-HDQ. Under these conditions a nearly stoichiometric relationship obtained between the binding of 6-HD and the number of sulfhydryl groups present on the protein either as free sulfhydryl groups or in latent form as disulfide bonds. This relationship and the failure of 6-HD to react with the amino groups of polylysine indicate that sulfhydryl groups are the primary sites of reaction.

The absolute requirement for oxygen, the ability of reducing agents such as ascorbic acid or sodium bisulfite to retard the reaction rate and the ability of sulfhydryl reagents like mercaptoethanol to interrupt the reaction of 6-HD with proteins strongly suggests that the reactive species is 6-HDQ. This quinone is an electron-deficient species and is highly reactive with nucleophiles [12]. Electrochemical studies have demonstrated that the reaction rate of 6-HDQ with compounds bearing free sylfhydryl groups is substantially faster than the alternate possibilities such as: (1) reduction of 6-HDQ to 6-HD, (2) internal nucleophylic attack resulting in the cyclization of 6-HDQ to 5,6-dihydroxyindole and (3) reaction with compounds bearing other nucleophilic groups like hydroxy or amino groups.

The discovery that 6-HD at 10^{-4} M or greater not only bound to various proteins but resulted in the formation of insoluble protein complexes [8] was most pertinent to the proposed relationship between the model system and the cytotoxic effect in vivo. Examination of the reaction products of 6-HD with various proteins by gel filtration and SDS polyacrylamide electrophoresis revealed a time-dependent formation of protein polymers of regularly increasing size. The products ranged from simple dimers, trimers, etc. to insoluble polymeric complexes with molecular weights in excess of 10^6.

These results indicate that after the initial covalent reaction between an available sulfhydryl group on a protein and one of the two electron-negative centers on the aromatic ring of 6-HDQ, reoxidation can be followed by reaction of a second sulfhydryl group with the remaining electron-negative center, thus forming a dimer of two peptide chains. This cross-linking reaction involving additional molecules of 6-HDQ proceeds until a complex protein lattice is generated. Proteins with only one or two sulfhydryl groups such as catechol-*O*-methyltransferase or human hemoglobin readily react with 6-HD but fail to undergo polymerization while horse hemoglobin with four sulfhydryl groups readily polymerized. The cross-linked complexes derived from bovine serum albumin, ovalbumin, and hemoglobin (horse), could not be dissociated by prolonged dialysis against various buffers, gel filtration, treatment with bisulfite or mercaptoethanol, treatment with 4 M urea and various detergents or by prolonged washing with either 0.4 N perchloric or trichloroacetic acid. Amino acid analysis, following hydrolysis of polymerized protein in 6 N HCl at 110°C for 24–72 h, revealed the presence of two new ninhydrin positive substances. Both of these substances contained radioactivity derived from the ^{3}H-labeled 6-HD used

initially to induce the cross-linking reaction. The major product was a strongly basic substance and the minor product was similar in chromatographic behavior to serine. It was tentatively concluded that the major basic product was a cysteinyl derivative of 6-HD in which the ethylamine side chain remained intact and the other minor product was a cysteinyl derivative in which the 6-HD portion had undergone intramolecular cyclization to an indole and thus lost its basic character. Thus, the irreversible nature of the interaction of the oxidation products of 6-HD with sulfhydryl groups on proteins in vitro and the subsequent cross-linking of these proteins is compatible with the proposal that this mechanism may underlie the cytotoxic action of 6-HD in vivo.

However, the direct extrapolation of the results of the interaction of 6-HD with proteins in vitro to the far more complex milieu of the sympathetic nerve terminal is unwarranted. Thus, a system more closely related to the conditions found in vivo was chosen to examine the cross-linking hypothesis for the mechanism of action of 6-HD. A clonal line of neuroblastoma cell, N1E115, was chosen for this purpose because it possesses a number of similarities to the adrenergic neuron including the presence of large, dense core vesicles which bind catecholamines [13]. Exposure of these cells to a non-lethal concentration of ^{3}H-labeled 6-HD resulted in a time-dependent, irreversible binding of 6-HD to various cell constituents and a dramatic change in the character of the intracellular proteins. Examinations of the treated cells revealed the presence of many high molecular weight proteins which were absent in preparations from untreated cells or in cells treated with dopamine. Virtually all of the ^{3}H-labeled 6-HD in the sample was irreversibly bound to these high molecular weight proteins. This evidence strongly suggests that 6-HD induced cross-linking of proteins in these cells and suggests an explanation of earlier reports of the toxic effects of 6-HD on a variety of cell cultures.

Another in vitro system, the isolated mouse atria, has been used to study the relationship between proposed mechanism of action of 6-HD and the cytotoxic effect in vivo. One of the earliest manifestations of the cytotoxic actions of 6-HD in vivo is a decrease in the neuronal uptake of norepinephrine. Pretreatment of mice with a cytotoxic dose (>1 mg/kg) of 6-HD results in a dose-dependent decrease in the uptake of norepinephrine in mouse heart measured in vivo [14] or in atrial preparations in vitro [15]. This decrease can be clearly demonstrated as early as fifteen minutes after administration of 6-HD. Studies with ^{3}H-labeled 6-HD demonstrated that as much as twenty percent of the amine taken up intraneuronally by the heart is covalently bound and that the time-course of the binding parallels the decrease in uptake of norepinephrine. In certain respects similar results have been obtained with isolated mouse atria exposed to ^{3}H-labeled 6-HD in vitro. The uptake and irreversible binding of ^{3}H-labeled 6-DA at a concentration of 10^{-5} M accumulated intraneuronally are nearly parallel for twenty minutes. The uptake of norepinephrine by atria under these conditions is unimpaired. Thus, it would appear that the covalent binding of 6-HD per se had failed to mimic the cytotoxic effect found in vivo. However, continued incubation of atrial preparations, which showed no further covalent binding of ^{3}H-labeled 6-HD, displayed a slow but progressive loss of the capacity to accumulate norepinephrine, either in the presence or absence of 6-HD, reaching a limit of approximately 45–50 percent after four hours. No loss in norepinephrine uptake was observed in control during this period. This slow but progressive decrease in the uptake capacity, which is independent of any further increase in the covalent binding of 6-HD, suggests that intraneuronal cross-linking of proteins may continue slowly following saturation of the binding sites for 6-HD. Direct attempts to characterize the formation of cross-linked proteins containing ^{3}H-labeled 6-HD, utilizing the same techniques mentioned earlier in the neuroblastoma study, was not satisfactory, presumably because of the relatively insignificant quantities of neuronal protein present in atria. However, at least five distinct proteins retaining isotope derived from ^{3}H-labeled 6-HD could be demonstrated on disc-gel electrophoresis. Thus, it may be possible through immunological techniques, utilizing antibodies specific for intraneuronal proteins, to isolate cross-linked protein polymers. While an absolute causal relationship between the covalent binding of the oxidation products of 6-HD to intracellular proteins and their subsequent cross-linking with the neuronal degeneration induced by 6-HD has yet to be established,

the present evidence strongly supports this view as the mechanism operative in vivo.

In conclusion, it is suggested that one rational approach for the design of cell-specific toxic agents can be patterned on the example of 6-HD. The first requirement in this approach, upon which the necessary specificity of the agent is dependent, involves finding a system which selectively permits the accumulation of the agent in a cell-type. In the case of 6-HD, the accumulation is a consequence of the uptake mechanism in the sympathetic neuronal terminal for the natural substrate, norepinephrine. The search for systems in other cell-types involves a knowledge of the substrate specificity of transport mechanisms and the affinity or quantitative relationships of substrates for a transport system. The second requirement in this approach is the design of an agent which has an affinity for the cell type and can undergo some spontaneous or enzymatically catalyzed change to give rise to a product or products with at least two sites for reaction with nucleophilic sites on proteins. By analogy to the example of 6-HD, the toxicity must be a consequence of the accumulation of a relatively high, critical concentration within the specific cell-type. The general, nonspecific interactions with protein at concentration below this critical level are not cytotoxic. Thus the types of agents which fit this rationale are not necessarily extremely toxic or reactive compounds.

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