Biochemical Pharmacology, Vol. 24, pp. 1929–1932. Pergamon Press, 1975. Printed in Great Britain.

COMMENTARY

ON THE THERAPEUTIC USE OF NUCLEOSIDES AND THE PENETRABILITY OF PHOSPHORYLATED COMPOUNDS

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Since nucleic acids are active participants in the development of pathology, e.g. virus infections and tumor growth, many natural and synthetic purine and pyrimidine bases and their nucleosides have been used by experimental and clinical therapeutists to affect this pathology. In the treatment of human disease, these analogues of nucleic acid components are frequently either less effective than one would hope or often more toxic than even a tightrope-walker-physician should use. In the former case, it is often found that the compounds are: (a) excreted, or (b) metabolized to inert or nonutilizable derivatives, or (c) that eventually an initially therapeutically effective compound is no longer active because of the emergence and selection of pathological cells that are unable to convert the chemical to an active metabolite.

Some problems in the use of D-arabinosyl nucleosides

Thus, a compound such as D-arabinosyl cytosine (ara-C), useful in the treatment of some tumors despite the attainment of transient and low blood levels, might not only be excreted via the kidney but also be deaminated to arabinosyl uracil, which is then readily cleaved to inactive fragments. Eventually a resistant tumor might even appear which is unable to phosphorylate ara-C to the 5′-nucleotide at a physiologically significant rate. To get the compound to the target for the proper length of time, the pharmacologist alters drug schedules and seeks ways of minimizing degradation by inhibiting degradative enzymes or by modifying the compound to protect it against degradation.

In the case of ara-C, although the clinicians have been testing various derivatives of the compound to increase the duration of its action, they have been unable to test in the clinic a combination of ara-C and a well defined inhibitor of ara-C degradation, tetrahydrouridine, which inhibits mammalian ara-C deaminase. For some years several investigators have been urging the Division of Cancer Treatment of the National Cancer Institute to support clinical tests of the combination of ara-C and tetrahydrouridine. A member of the Division has asserted that "when an agent is ineffective alone, its use in combination trials is generally unwarranted" [1]. The adoption of this rule would preclude the use of a combination comprised of an effective agent and a nontoxic substance which minimized the excretion or degradation of the agent; this author has recently requested a clarification of the scope of such a statement and its intent via *Cancer Chemotherapy Reports* [2].

We have been studying the mode of action and lethality of arabinosyl nucleosides since 1956 and consider the problem of increasing their therapeutic effectiveness to be important. Since 1961 we have been concentrating on problems relating to the toxicity, mode of action, and metabolism and utility of D-arabinosyl adenine (ara-A), whose biological activity we first detected. During the last decade, while we have been observing inhibitory effects of ara-A nucleotides on enzymes such as DNA polymerase, ribonucleotide reductase and adenyl cyclase, we have noted a growing parallel literature which has described an inhibitory activity of ara-A in various virus infections and in some animal tumors. The compound has had an unusually low toxicity, which seemed promising, but also often had only marginal activity in some animal systems. Both effects have been attributed to metabolic degradation, because ara-A is readily deaminated by adenosine deaminase.

Some effects of an inhibitor of adenosine deaminase

We have developed two approaches to increasing the therapeutic efficacy of ara-A. A recent report of a new, powerful and relatively nontoxic inhibitor of adenosine deaminase [3] has led us to study combinations of ara-A and this deaminase inhibitor. Comparing the combination to ara-A alone, we have observed a marked increase (at least 20-fold) of the lethality of the combined drugs to mammalian cells in tissue culture, as well as an increase in the survival of tumor-bearing mice [4]. Furthermore, the deaminase inhibitor markedly potentiated the activity of other therapeutically marginal adenine nucleosides, such as cordycepin and D-xylosyl adenine [4]. It is evident then that, even as in the case of ara-C and tetrahydrouridine, the protection of the adenine analogues against deamination may sharply enhance the inhibitory activity of numerous well known adenine nucleosides and may significantly enlarge our potential therapeutic armamentarium.

* The author is an American Cancer Society Professor of Microbiology. The work of his laboratory on the subject described in this Commentary was supported initially by a grant from the American Cancer Society (E 587) and more recently by Grant 1 R01 11636 from the National Institute of Allergy and Infectious Diseases.

Penetration of nucleotides in animal cells

Our second approach to the problem of the preservation of adenine nucleosides *in vivo* has been directed to the test of a shibboleth of biochemical pharmacology, namely the view that nucleotides do not enter cells. This assertion rested in large part on some experiments of the 1950's. In 1960 we contributed to this dictum ourselves by the demonstration that intact *Escherichia coli* quantitatively excludes certain pyrimidine nucleotides, although bacteria can dephosphorylate the nucleotide on the outside of the membrane and rephosphorylate the derived nucleoside on the inside [5]. It was demonstrated at that time that the nucleotide, fluorodeoxycytidylate, had a more prolonged lethality to *E. coli* than did the nucleoside, fluorodeoxycytidine. Deamination and cleavage of this nucleoside to the bacteriostatic fluorouracil rapidly arrest the lethal action of the nucleoside. On the other hand, administration of the nucleotide and the conversion of fluorodeoxycytidylate to the intracellularly lethal fluorodeoxyuridylate via a slow dephosphorylation and deamination followed by rephosphorylation within the cell maintain the lethal activity of the toxic fluorodeoxyuridine. It was in fact suggested that this method of administration of a nucleoside, i.e. as a nucleotide, might preserve the nucleoside for prolonged therapeutic effects [5]. There is no indication that this proposal was ever tested carefully in animals.

In any case, several years ago we observed that, at concentrations at which the toxicity of ara-A is relatively low in tissue cultures of mouse fibroblasts, the 5′-nucleotide, ara-AMP, is significantly more lethal. A more careful analysis of this observation has shown that the toxicity of the nucleotide is expressed more slowly, but leads to a far more extensive reduction of viable cells [4]. The toxicity of ara-A disappears in a day, when ara-A is totally deaminated, whereas ara-AMP, which is not deaminated, continually bathes the cells. Tests with doubly labeled ara-AMP [6] have revealed that, in contrast to our bacterial study, the nucleotide does in fact penetrate animal cells, is converted to ara-ATP at intracellular concentrations inhibitory to mammalian DNA polymerase, and enters DNA in internucleotide linkage [6]. The nucleotide enters the cells at rates only 3–5 per cent of the apparent initial rate of entry of ara-A. Although some of the exogenous nucleoside is converted intracellularly to toxic ara-ATP, most of the ara-A is deaminated and ejected as the relatively inactive arabinosyl hypoxanthine; in contrast, very little of the exogenous nucleotide is detoxified intracellularly. Our result on nucleotide penetration may be of significance because of the report that humans are relatively low in kidney phosphatase and that ara-AMP is not immediately dephosphorylated in this species [7].

The rate of penetration of the nucleotide is so slow that we needed labeled compounds of quite high specific activity to detect this phenomenon. Examination of the early experiments of Roll and of Heidelberger indicated that, at the specific activities used, these workers could not have detected such low rates of penetration. In one group of experiments with AMP, Liebman and Heidelberger [8] had actually obtained some indication that a small fraction of the nucleotide had entered the cell as such. Most investigators chose to focus on the degradation of the nucleotide and the redistribution of its components, and this study was generally interpreted as proof of exclusion of nucleotides. We can say that the initial lead in our experiments, which determined the design of the experiment on isotope incorporation and compelled our perseverance, was the observation that a nucleotide was more lethal than a nucleoside at low intracellular concentrations.

Having observed that nucleotides do enter cells, albeit slowly, and having re-examined the literature, we have come to feel that the existence of the dictum, i.e. "that nucleotides do not penetrate," is a powerful inhibitor of research in this area. Indeed we did not test certain nucleotides available to us earlier, presumably as a result of the subliminal inhibition induced by the exclusion rule. More recently we have tested 2′,3′-dideoxyadenosine 5′-phosphate, the nucleoside of which is lethal to bacteria but which is almost inactive in mouse fibroblasts. We have now found that the nucleotide is quite lethal to the fibroblasts [4] and imagine that the exogenous nucleotide can be used to terminate DNA in animal cells, even as the nucleoside does in bacteria.

On the penetrability of phosphorylated compounds in procaryotic and eucaryotic cells

A re-examination of the literature also suggests that the exclusion rule has been extrapolated incorrectly to other areas of pharmacological interest. Thus there are few reports of the systematic test of phosphorylated compounds, although there are numerous reports of the biological activity of such compounds when supplied to cells. (I will close this Commentary with a brief summary of such reports.)

Nucleic acids

It is customary to contrast the uptake of nucleic acids by cells with the non-entry of nucleotides. However, in many instances uptake in animal cells has been followed by a very sensitive biological test, i.e. infection. In recent years the inference of penetration has been confirmed by cytological, isotopic and chemical tests, although the mechanisms of these penetrations are quite obscure and indeed essentially without study. Our knowledge of bacterial transformation by DNA is 30 years old, infection of bacteria by various viral nucleic acids is common enough (even better and much less host-specific in spheroplasts), and one may even isolate bacterial mutants unable to grow in the absence of exogenous tRNA. The mechanisms of these phenomena are still mysterious, and with the exception of some small insights on the nature of competence in transformation, without study. It is known that this uptake can be sharply increased by treatment of the cells with polycations. The uptake by eucaryotic cells is occasionally ascribed to pinocytosis and its stimulation, but certainly this mechanism has never been systematically explored as an approach to increasing the uptake of difficultly penetrable potential chemotherapeutic agents.

Organelles and cells

It is known that isolated organelles such as nuclei, mitochondria and chloroplasts will incorporate various exogenous nucleoside triphosphates into internal nucleic acids at significant rates. On the other hand, studies on energy production in mitochondria have revealed the presence of relatively specific translocases for adenosine polyphosphates. Virtually nothing has been done to attempt to understand the relation between the two types of studies. Nevertheless, it appears that the relative exclusion of nucleotides at the mammalian cell surface is not inherent to all membranous structures. In these terms then, the nature of the possibly specific exclusion mechanisms at the cell surface is a curiosity and a problem, perhaps related to the need to exclude potentially disturbing agents such as the nucleic acids and nucleotides. This defense mechanism is undoubtedly strengthened by the additional presence of nucleases and nucleotidases at the surface of some cells. Obviously, however, our mouse fibroblasts (L cells) lacked a significant amount of such a nucleotidase active on 5′-ara-AMP, so that this defense is either not ubiquitous or is sufficiently specific to permit evasion by appropriate analogues. Furthermore, the need for pinocytosis or ingestion or both, as well as relatively specific transfer systems, provides additional potential avenues for bypass of the existing defenses.

Poking holes in cells

In the last few years, the permeability of bacteria and eucaryotic cells has been increased by treatment with toluene or Ca^{2+}, plasmolysis, low temperature, etc. Such cells have been useful in facilitating the incorporation of nucleotide metabolites, even nucleoside triphosphates, in approaches to the study of a synthesis *in situ* of the nucleic acids. Although in many instances the damage to the membrane by such treatments has been irreparable, it is not clear that this would be so after treatments with such antibiotics as the polyenes and tunicamycin, which distort normal membrane structure and can be expected thereby to induce an increased permeability to many substances.

Cyclic nucleotides

The pharmacological activities of exogenous cAMP and cGMP in cell cultures are essentially taken for granted. The existence of cAMP-requiring mutants in bacteria is well known, and the numerous effects of exogenous cAMP in these cells are noted, but not pursued, to determine if the compound merely acts upon the external membrane. Similar questions can be posed for this substance concerning its effects on animal cells. A very few studies do appear to indicate that the nucleotide does in fact enter the cells, although these experiments are not as chemically rigorous as one would like. Further, at least two studies suggest that the avian erythrocyte and *E. coli* may be able to pump the compound out against a concentration gradient. The appearance of nucleotides in the culture media of many bacteria is a common phenomenon, but it is not evident in most of these instances whether this is due to secretion or damage to the bacterial membrane.

Organic sulfates and phosphates

Many cells have devised mechanisms for the transport of strong anions, including phosphate and sulfate. That phosphorylated compounds and other strong anions do enter bacteria is affirmed by the existence of strains of *E. coli* or *Neisseria gonorrhoeae* which are unable to use thiamine and require thiamine pyrophosphate for growth. Some Pseudomonads cultured on glucose-6-*O*-sulfate will excrete gluconate 6-*O*-sulfate. Others can generate choline-*O*-sulfate intracellularly and excrete this compound. In the utilization of choline-*O*-sulfate by Neurospora, the compound penetrates intact and is hydrolyzed intracellularly to liberate utilizable sulfate.

In the early 1950's, the competition experiments of Roberts and Roberts [9] demonstrated that glucose 6-phosphate and fructose 6-phosphate enter *E. coli* intact. This fact has been rediscovered and elaborated upon in the last decade, as a result of which we now know of transport systems in *E. coli* for hexose 6-phosphates operative on pentose and heptulose phosphates, and also of transport systems for glucose-1-phosphate and L-glycerophosphate. Indeed the uptake of the phosphorus-containing antibiotic, phosphonomycin, into bacteria is dependent on the transport system for the latter. Further, several phosphonate analogues of glycerophosphate and other triose phosphates have been found to be effective inhibitors of various triose-related metabolic pathways in *E. coli*. Phosphonoacetate has recently been found to be an interesting antiviral agent in animal cells and animals. Innumerable investigators test the uptake of sugars in eucaryotic cells, but I have never seen a description of a comparable test in such cells with a sugar phosphate, much less a charged analogue of this potential metabolite. Is this neglect a consequence of the pervasive influence of the exclusion rule?

Observations that need some work

Some evidence has been obtained for the apparent penetration of nucleotides or of their derivatives into bacteria, protozoa and plants. For example, aminoalkyl adenylates (aa ol-AMP), which are potent inhibitors of aminoacyl-tRNA synthetases, inhibit the growth of strains of *E. coli* in a manner reversible by addition of the specific amino acid. Exogenous AMP markedly stimulates uracil incorporation into RNA and glycogen synthesis in Tetrahymena. UDP-glucose and GDP-glucose are good substrates for the synthesis of polysaccharide by the epidermal hair cells of the cotton plant. A review of the literature suggests that it might be useful to explore the direct incorporation of tubercidin 5′-phosphate on schistosomes or of adenylate analogues on trypanosomes. If these biological and biochemical effects can be confirmed, it would be of interest to determine if these compounds do penetrate intact.

Viable mutants of yeast have been isolated which incorporate exogenous doubly labeled thymidine 5′-monophosphate into DNA. The specificity of the surface change produced by these mutations has not yet been described, but it is evident that the changes do not lead to a general disorganization or severe damage of the surface components. The importance

of understanding and learning how to manipulate these types of chemical changes in yeast is obvious.

RNA and adenylate, as well as adenosine, are reported to have insulin-like activity on adipose tissue in increasing the utilization of exogenous glucose or in lipogenesis. Periodate-oxidized adenylate inhibits nucleic acid synthesis, particularly that of DNA, in Ehrlich tumor cells to a greater extent than does periodate-oxidized adenosine. Reference may also be made to the studies of Wigler who demonstrated toxicities in cell culture systems for the mono-ionized nucleotide analogues, fluorodeoxyuridine 5′-sulfate and bromodeoxyuridine 5′-methyl phosphonate. In all of these experiments direct evidence of penetration of the nucleotides was not given. However, it has been reported recently that uridylyl-2′,5′-[^{14}C]adenosine enters rabbit red cells without enzymatic or chemical hydrolysis. Unfortunately, in that paper, quantitation is provided in cpm but not in molecules.

The 3′,5′-cyclic phosphates of various nucleosides have been shown to have potent antitumor activities, some at sites not sensitive to the usual antitumor agents. Some of these, such as 3′,5′-cyclic ara-AMP, are cleaved by cellular phosphodiesterases and so may be active as the 5′-nucleotide, whose toxic action it mimics very closely. In our hands, 3′,5′-cyclic ara-AMP and 5′-ara-AMP give identical prolonged killing curves with L cells.

Conclusions

From this summary it would appear that the rule concerning the exclusion of nucleotides and their derivatives requires some modification. Nucleotides are stable and more soluble derivatives of nucleosides which may facilitate administration of the latter and permit their greater survival in a human under therapy. Some nucleotides can actually enter cells as such, although at low rates. Nevertheless, the toxicity of some analogues permits the detection of even low rates of penetration and perhaps even permits the direct utilization of nucleotides in therapy. It is possible that systematic study of the mechanisms of penetration will help to increase the rate of such penetration and even of its specificity. Since it has been possible to obtain activities with some nucleotides undetectable with their respective nucleosides, it is conceivable that the 5′-phosphates of some other nucleosides, previously thought to be inactive, may prove to have interesting toxicities.

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