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COMMENTARY

Selective Depletion of DNA Precursors

AN EVOLVING STRATEGY FOR POTENTIATION OF DIDEOXYNUCLEOSIDE ACTIVITY AGAINST HUMAN IMMUNODEFICIENCY VIRUS

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ABSTRACT. Human immunodeficiency virus type 1 (HIV-1) is wholly dependent on its host cell for a variety of essential metabolites. Among the latter are the deoxynucleoside-5′-triphosphates (dNTPs) required for reverse transcription of the single-stranded RNA viral genome into double-stranded viral DNA. Since viral DNA synthesis has an absolute requirement for all four dNTPs, restriction of a single one of these is sufficient to inhibit HIV-1 replication. To date, this therapeutic strategy has been most successful when depletion of the individual dNTP is coupled with exposure to its corresponding chain-terminating dideoxynucleoside (ddN). While several examples of such combined therapy have been defined and studied *in vitro*, that which has been investigated most extensively at both the laboratory and the clinical level is ddATP exposure combined with dATP depletion [with dATP restriction being induced by the ribonucleotide reductase inhibitor hydroxyurea (HU) and ddATP generated from its prodrug 2′,3′-dideoxyinosine (ddI)]. Several long-term clinical trials of the hydroxyurea/2′,3′-dideoxyinosine combination have been completed, with plasma viral RNA being reduced to undetectable levels in a substantial fraction (one-third to one-half) of the patients treated. The major advantages of this and analogous combinations discussed in this review are their low cost relative to other current multiple drug protocols and their potential for retention of activity against drug-resistant HIV mutants. BIOCHEM PHARMACOL **55**;10:1551–1556, 1998. © 1998 Elsevier Science Inc.

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HIV-1§, lacking the capacity to generate dNTPs for reverse transcription, is wholly dependent on its host cell for these essential substrates. Selective restriction of deoxynucleotide supply, without irreversibly impairing host-cell DNA synthesis, is thus an obvious target for the control of HIV-1 replication.

An early observation that DNA virus replication could be slowed or even stopped by specific inhibitors of deoxynucleotide biosynthesis was made by Slabaugh and her co-workers [1], not with HIV-1, but with vaccinia virus, and employing the ribonucleotide reductase inhibitor HU. Vaccinia, unlike HIV-1, can encode its own ribonucleotide reductase subunits; the latter, however, are closely homologous to their mammalian counterparts [1]. These investigators showed that virus growth was strongly inhibited at a concentration of 1 mM of HU and blocked entirely at 2 mM. In addition, the important observation was made that the inhibitory effect could be reversed by 2′-deoxyadenosine (if the latter were protected from enzymic deamination), but not by the deoxynucleosides deoxycytidine, thymidine, or deoxyguanosine. Compatible with this observation was the finding that the dATP pool, although not the smallest of the four dNTP pools [2], was the most susceptible to depletion by HU. Similar observations on inhibition of Moloney murine leukemia virus replication by HU have been made more recently by Goulaouic and coworkers [3]; these authors further noted that the HU effect on replication of this retrovirus depended on reduction of intracellular dNTP pools, rather than on the well-known ability of HU to bring about cell-cycle arrest, because the polymerase α inhibitor aphidicolin, which, like HU, produces a block at the G_1/S border, failed to affect the reverse transcription step.

ANTI-HIV-1 EFFECTS OF HU AND OF HU/ddN COMBINATIONS

The first studies specifically directed toward the possible application of HU in restricting HIV-1 replication appear

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§ *Abbreviations:* HIV-1, human immunodeficiency virus type 1; dNTPs, 2′-deoxynucleoside-5′-triphosphates; HU, hydroxyurea; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; RT, reverse transcriptase; ddNTPs, 2′,3′-dideoxynucleoside-5′-triphosphates; ddNs, 2′,3′-dideoxynucleosides; ddI, 2′,3′-dideoxyinosine; AZT, 3′-azido-3′-deoxythymidine; ddC, 2′,3′-dideoxycytidine; D4T, 2′,3′-didehydro-3′-deoxythymidine; 3TC, 2′-deoxy-3′-thiacytidine; IMP, inosine-5′-monophosphate; and ddG, 2′,3′-dideoxyguanosine.

to have been carried out in 1993/1994 by Gao and co-workers [4] and by Meyerhans *et al.* [5], both groups using the PBMC system. In both of these studies, a significant anti-HIV-1 effect was demonstrated, although at comparatively high HU concentrations (1–3 mM); Gao and his coworkers showed that HU treatment of PHA-activated HIV-1-infected PBMCs resulted in inefficient and incomplete HIV-1 DNA synthesis, similar to that seen in quiescent PBMCs, with no HIV-1 progeny being produced. On the basis of these studies, both groups suggested that HU, being noncytotoxic in the *in vitro* PHA/PBMC system, could have clinical applications if used alone as an anti-HIV-1 agent.

While the latter prediction was subsequently found to be over-optimistic [6, 7], the observation made by these workers that, in the PHA/PBMC system, as in the vaccinia virus system, dATP pools were more severely depleted by HU exposure than were those of the other dNTPs, and the demonstration that inhibition of HIV-1 DNA synthesis produced by HU could be reversed by deoxyadenosine [5] (a further indication that, as with the vaccinia and Moloney viruses, HU was acting through its ability to inhibit host-cell ribonucleotide reduction, rather than by some alternate mechanism such as a direct effect on the virus itself), were soon found to be of key significance. It had long been recognized that the anti-HIV-1 activity of ddNs as RT inhibitors and viral DNA chain terminators does not depend solely on the absolute level of the ddNTP generated intracellularly, but rather on the ratio of the concentration of the latter to that of the corresponding physiological dNTP (i.e. ddCTP/dCTP, ddATP/dATP etc.). Thus, a pharmacologically induced decrease in the level of the corresponding dNTP could, in principle, have as great an antiviral effect as an increase in the ddNTP concentration. In 1994, in *in vitro* studies of HU/ddN combinations, three groups of investigators showed that the anti-HIV-1 effect of ddI (the prodrug of ddATP), was potentiated markedly by the dATP-depleting agent HU, whereas much lesser potentiation by HU was noted for the ddNs AZT and ddC [8–10]. Furthermore, as a potentiating agent for ddNs, HU was effective at much lower concentrations than when used alone (i.e. 50–100 μM, rather than 1–3 mM), an observation that would later be seen to have clinical significance.

On further study, utilizing the PHA/PBMC test system, a clear difference emerged between the mechanism for HU potentiation of the purine-based ddN ddI and that for the pyrimidine-based ddNs AZT and ddC. In the case of ddI, there was no increase in the intracellular concentration of the active metabolite (ddATP), but, as would be anticipated from previous studies with HU, a substantial drop in dATP, yielding a 2.4-fold favorable shift in the ddATP/dATP ratio (at 0.1 mM of HU) [11]. For AZT and ddC, however, the corresponding 5′-triphosphates (AZTTP and ddCTP) increased in concentration, partially counterbalanced by somewhat lesser increases in dTTP and dCTP, resulting in much less favorable shifts in ddNTP/dNTP ratios (1.1- to 1.5-fold) and lesser potentiation of anti-HIV activity than was seen with ddI. When the activities of the salvage enzymes thymidine kinase and deoxycytidine kinase, which are responsible for the initial phosphorylation of AZT and ddC, were assayed, it was found that each had increased several-fold after a 24-hr exposure to HU (0.1 mM), an effect apparently due to the well-known action of low-dose HU in prolonging the duration of the S-phase of the cell cycle (partial synchronization): thymidine kinase is a cell-cycle regulated enzyme, with its rate of synthesis being highest in S-phase, while deoxycytidine kinase, although not cell-cycle regulated, increases in activity during S-phase in many cell systems, apparently because of post-transcriptional regulation [12, 13]. The phosphorylation of ddI, which is catalyzed by a cell-cycle independent enzyme [14], was not affected by HU.

CLINICAL STUDIES WITH HU/ddI

The first clinical study of the HU/ddI combination was reported by Vila and his coworkers in 1995 [15], with a 1-year follow-up report appearing in 1996 [16]. The initial study cohort comprised 12 patients who had not received ddI therapy previously, and who had $CD4^+$ counts in the range of 263–582 mm^2; the study was later expanded to include 25 patients. The doses used were 200 mg ddI and 500 mg HU, both given twice daily. All patients showed a drop in plasma viral load, and in 13 of 24 patients evaluated at 6 months and 10 of 20 patients evaluated at 1 year, plasma HIV-RNA was undetectable. Lymph node biopsies were carried out on 7 patients; in 5 of these, no virus was detectable in lymph node mononuclear cells. In another recently published study, comprising a smaller and more heterogeneous group of HIV-1-infected patients, the mean decrease in plasma viral load was 2.1 log after an average of 62.5 weeks of treatment, with plasma viremia falling below the detectable level in 3 out of 6 patients [17]. In both groups of patients, the treatment was well-tolerated; despite the sustained reduction in viral load, however, little consistent improvement was noted in $CD4^+$ counts, and in the French study [16], 4 of 25 patients developed a reversible leukopenia, both likely cytostatic effects of HU at the 1000 mg/day dose-level.

In addition to the unusually large magnitude of the decrease in plasma viral RNA, a second striking result of these and subsequent clinical studies was the frequent absence of viral "rebound." Vila and his co-workers reported recently that, in 2 patients with early-stage HIV infection who had been treated with HU/ddI for 1 year and for whom all antiviral treatment was then suspended, no HIV RNA was detectable in plasma, PBMCs, or lymph node mononuclear cells 1 year later [18]. Proviral DNA, incompetent for transcription activity (and, therefore, for the release of infectious virions) was still detectable at very low levels in lymph nodes of both patients. Extension and confirmation of these studies will be awaited with interest. Lori and his coworkers [19, 20] have observed that in patients from whom virus could still be recovered, the

ddI/HU combination, despite bringing about a marked decrease in the rate of viral replication, did not prevent the emergence of mutant virus, including virus exhibiting the characteristic L74V mutation associated with ddI resistance [21, 22]; mutations in the 69–74 region appeared to develop more frequently in patients receiving the ddI/HU combination than in patients receiving ddI alone.

This apparent increase in mutation rate would appear to be a predictable consequence of dATP depletion, since it has long been known from DNA replication studies in model systems that unbalanced dNTP levels at replication sites lead to an increased frequency of nucleotide substitution errors [2]. Compounding the dNTP pool imbalance-induced mutagenesis in the case of HIV-1 is the circumstance that HIV-1 RT, unlike host cell DNA polymerases, lacks proofreading capacity. The retention of clinical susceptibility to ddI in the presence of HU could be attributable to simple enzyme kinetic considerations at the putative dNTP and ddNTP binding site, thought to be located in the palm subdomain of the p66 subunit of RT [23]: although the K_i values for ddATP increase as much as several-fold in ddI-resistant RTs [22, 24], the expected decrease in pharmacological activity could, in principle, be negated by lessened competition from the lowered pools of the corresponding physiological deoxynucleotide (dATP), even without a change in the kinetic parameters of the latter as a substrate for RT. Correlations between *in vitro* drug resistance, general clinical status, and clinically observed viral load have never been established for ddI [25–27], and further study of the long-term antiviral activity of the HU/ddI combination would thus appear to be warranted at both the clinical and laboratory levels. It would appear, also, that the initial concern that the effectiveness of the strategy of selective depletion of viral DNA precursors in conjunction with the administration of the appropriate ddN anti-HIV-1 agent would rapidly become ineffective as ddN resistance mutations developed may be unwarranted in the case of the HU/ddI combination.

THREE-DRUG COMBINATION STUDIES WITH HU

Several clinical investigators have studied the triple combination HU/ddI/D4T. Rutschmann and his coworkers [28] compared the combination ddI + D4T + placebo versus ddI + D4T + HU in 100 moderately immunosuppressed HIV-1-infected patients. When the two groups were compared at 3 months, plasma viral RNA had decreased to undetectable levels in 55% of patients receiving the triple combination and in 32% of patients receiving only ddI and D4T. Similar results with this triple drug combination were reported in a study by Rossero and his coworkers [29]. Whether the addition of D4T to the HU/ddI combination results in increased long-term anti-HIV-1 effects beyond those seen with HU/ddI alone has not been determined and will require further clinical study. In an *in vitro* study in MOLT-4 cells, HU (50 μM) increased the conversion of D4T to D4TTP by several-fold, with a concomitant decrease in dTTP pools of 28% [30]; as we and others have noted, however, the effect of low-dose HU on dTTP pools varies among cell lines, with increases in dTTP having been reported in PHA/PBMC and in CEM cells [11, 31].

HU/AZT STUDIES

In an early study, Karlsson and her co-workers [32] showed that even low levels of HU (50–200 μM) increased the phosphorylation of both AZT and the experimental anti-HIV-1 agent 3′-fluorothymidine by several-fold in CEM cells. dTTP pools were also increased, although to a lesser extent. These investigators did not examine the antiviral effect in HIV-1-infected cells, but suggested the potential clinical applicability of these two HU/ddN combinations. In a related but much more recent study [33], this investigation was extended to HIV-1-infected cells, and PBMC as well as CEM cells were utilized. HU strongly potentiated the activity of AZT in both cell types. The effect of HU on the widely used combination of AZT and 3TC has also been examined recently in CEM cells [34]. The concentration of HU used was moderately high (1 mM). Anti-HIV-1 activity was not measured, but increased phosphorylation of both drugs was observed. dTTP pools were increased, but no change was noted in dCTP pools.

In a recent clinical study comparing the efficacy of the HU/ddI combination with that of the HU/AZT combination, HU/AZT, unlike HU/ddI, failed to show any advantage over AZT or ddI monotherapy [35]. While speculation concerning this differing effectiveness based solely on laboratory studies is hazardous, a possible drawback of the HU/AZT combination has been alluded to above, i.e. although HU undoubtedly increases the phosphorylation of AZT, it also (in some experimental systems) increases the level of the competing dNTP, dTTP, thus partially negating the anti-HIV-1 effect. In addition, for single-agent AZT, unlike ddI, a positive correlation appears to have been well established between the development of drug resistance and the deterioration of clinical status [36–38].

OTHER RIBONUCLEOTIDE REDUCTASE INHIBITORS

While HU has received the most detailed study, other dNTP-depleting agents have been examined for their potential use in anti-HIV therapy. In *in vitro* studies, Reichard and his coworkers [39, 40] have investigated the properties of the interesting compound 2′-deoxy-2′-azidocytidine; this nucleoside analogue is phosphorylated by deoxycytidine kinase, an enzyme highly expressed in lymphoid cells, yielding the active form of the drug, a ribonucleotide reductase inhibitor with effects on ddN phosphorylation and on dNTP pools similar to those of HU [40]. A major potential advantage of 2′-deoxy-2′-azidocytidine would be its greater specificity for lymphoid tissue, the

major site of HIV replication. A possible drawback would appear to be that while HIV replicates primarily in cells of the lymphoid system, there may well be additional replication sites where anti-HIV drugs can penetrate only with difficulty and that can therefore act as "sanctuaries" for the virus [41–43]. Among these are the HIV-harboring cells of the central nervous system, where penetration of cytidine analogues is extremely slow [44]. In this respect, HU, with its wide and rapid distribution to all tissues, including brain [45], would appear to offer advantages over 2′-deoxy-2′-azidocytidine and most other ribonucleotide reductase inhibitors.

OTHER DNTP-DEPLETING AGENTS

A number of investigators have utilized IMP dehydrogenase inhibitors such as mycophenolic acid, ribavirin, and tiazofurin, in combination with ddNs. These compounds have the dual properties of increasing intracellular pools of IMP, the 5′-phosphate donor for purine-based ddNs such as ddG, carbovir, ddI and 2′-β-fluoroddA, and of depleting dGTP levels (by inhibition of GTP synthesis through the IMP → XMP → GMP → GTP pathway). In early studies, we found the anti-HIV activity of ddG to be potentiated up to 6.5-fold by these agents in the H9 test system [46], and Vince and coworkers reported synergism between ribavirin and the carbocyclic analogue of 2′,3′-didehydroddG (carbovir) [47]. These studies have not yet been extended to ddG-resistant or carbovir-resistant lines. In a recent study in several cell lines, including PHA/PBMCs and monocyte/macrophages, Ichimura and Levy [48] described anti-HIV activity of mycophenolic acid as a single agent, a property likely due to the ability of low levels of this compound to deplete cytoplasmic GTP (and hence dGTP) without eliciting host-cell cytotoxicity. Ribavirin and tiazofurin are without anti-HIV activity as single agents, apparently because of the unrelated cytotoxicity seen when these compounds are utilized at concentrations sufficient to lower dGTP levels appreciably [46].

Even though dATP pools are relatively abundant in eukaryotic cells (averaging about twice those of dGTP [2]), this deoxynucleotide would appear to be the most susceptible of the four dNTPs to selective depletion, probably because of the ubiquitous presence of adenosine deaminase and thus the relative unavailability of substrate for direct phosphorylation of deoxyadenosine to dAMP and thence to dATP. Consequently, the supply of this dNTP depends primarily on *de novo* pathways and ribonucleotide reductase. For other deoxynucleotides, salvage pathways are active, and dNTP pools are accordingly more resistant to pharmacological manipulation at the ribonucleotide level. ddI and other inosine- or adenosine-based ddNs would thus appear to be better suited than other ddNs for potentiation by this mechanism.

Despite these considerations, however, successful preliminary attempts have been made *in vitro* to enhance the activity of thymidine, deoxyguanosine, and deoxycytidine-based ddNs against both wild-type HIV and drug-resistant mutant lines by pharmacological depletion of the corresponding dNTP. For example, in studies with an infectious recombinant HIV-1 clone containing the Q151M substitution, a mutation that had been observed previously in patients receiving long-term AZT/ddI therapy, and who exhibited varying degrees of resistance to AZT, ddC, ddI, ddG, and d4T [49], it was observed that depletion of dTTP with thymidylate synthase inhibitors increased sensitivity to AZT more than 10-fold [50]. Further *in vitro* study will be required to determine the potential usefulness of these and similar combinations.

PROJECTIONS AND SPECULATION

Despite its relatively low cost and its ability to reduce viral load to undetectable levels in a large proportion (30–55%) of patients, the ddI/HU combination would not appear likely to supplant currently used three-drug combinations with their somewhat higher response rates, except in special circumstances such as cases where HIV-protease inhibitor resistance or intractable drug idiosyncrasy has developed. Nevertheless, ddI/HU and related dNTP-depleting combinations would appear to warrant further exploration at both the laboratory and clinical levels. First, the complete range of such combinations has not yet been explored, and other combinations may well yield results superior to ddI/HU [51]. Second, further study of the original ddI/HU combination may yield information permitting improvement of present response rates; for example, do patients who show partial rather than complete responses have higher endogenous intracellular dATP levels, and, if so, are superior alternative methods for lowering dATP pools available? Could the lymphocytopenia noted in some patients be avoided by lower doses or altered scheduling of HU, or by the simultaneous administration of appropriate lymphocytokines? Third, HIV-1 replication is not only wholly dependent on the host cell for dNTPs and other metabolites, but appears susceptible to HIV-suppressive C-C chemokines generated by other cell types such as $CD8^+$ T-cells [52–56]. Would depletion of host cell dNTPs, combined with administration of HIV-suppressive cellular factors, result in total eradication of the virus? Resolution of these and similar questions may well permit the optimal use of this promising treatment modality as a supplement or even as an alternative to existing modes of anti-HIV therapy.

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