Anti-HIV drugs decrease the expression of matrix metalloproteinases in astrocytes and microglia

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Summary
The introduction of potent antiretroviral drugs for the treatment of patients with human immunodeficiency virus (HIV) infection has dramatically reduced the prevalence of HIV-associated neurological disorders. Such diseases can be mediated by proteolytic enzymes, i.e. matrix metalloproteinases (MMPs) and, in particular gelaatinases, released from glial cells. The aim of this study was to investigate whether the antiretroviral drugs commonly used for the treatment of HIV-infected patients modulate the activity of MMPs in astrocyte and microglial cultures. Primary cultures of rat astrocyte and microglia were treated with different doses of zidovudine (AZT) or indinavir (IDV) for 20 h and simultaneously activated by exposure to lipopolysaccharide (LPS). Culture supernatants collected from astrocytes and microglia after 24 h incubation were subjected to gelatin zymography and western blot analysis for the assessment of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) protein levels. Total RNA was extracted from glial cells and used for reverse transcriptase–polymerase chain reaction for the assessment of mRNA expression. Our results indicate that both astrocyte and microglial cells constitutively express MMP-2 mRNA and protein. LPS treatment increased MMP-2 mRNA and protein expression in astrocytes, but not in microglial cells. The treatment with both AZT and IDV dose-dependently inhibited the expression of MMP-2 in astrocytes, whereas it had no effect on microglial cells. The expression of MMP-9 in both astrocytes and microglia was induced by LPS treatment and was dose-dependently inhibited by AZT and IDV treatment in LPS-stimulated astrocytes and microglia. These results raise the possibility that AZT and IDV interfere directly with MMP production in glial cells and independently from their antiviral activity, thus suggesting the possible therapeutic use in neurological diseases associated with MMPs involvement.

Keywords: matrix metalloproteinases; antiretroviral drugs; HIV dementia; microglia; astrocytes

Abbreviations: AZT = zidovudine; BBB = blood brain barrier; DMEM = Dulbecco’s modified Eagle’s medium; GFAP = glial fibrillary acidic protein; HIV = human immunodeficiency virus; HIVD = HIV-associated dementia; IDV = indinavir; LPS = lipopolysaccharide; MMP = matrix metalloproteinase; MBP = myelin basic protein; PA = 1,10 phenanthroline; RT–PCR = reverse transcriptase polymerase chain reaction; TIMP = tissue inhibitors of matrix metalloproteinase.


Introduction
The late stages of human immunodeficiency virus-1 (HIV) infection are frequently complicated by a variety of neurological disorders including opportunistic infections, neoplasms and subcortical dementia. The latter can be ascribed to the direct invasion of the CNS by HIV. The introduction of potent antiretroviral treatment has induced a dramatic reduction in the prevalence of neurological disorders in patients with HIV infection. The best studied neurological disease is HIV-associated dementia (HIVD) which, despite the extensive availability of highly active antiretroviral therapy (HAART), still affects a significant number of HIV-infected patients and is a major cause of disability. Clinically, HIVD is characterized by impaired memory and concentration, and psychomotor slowing (Price, 1996). Histological examination
of the brain frequently shows that the subcortical white matter and nuclei grigi profondi are the main affected cerebral structures, and subcortical/cortical atrophy and demyelination are the main characteristics of HIVD (Dal Canto, 1989; Price, 1996).

Studies aimed at evaluating the pathophysiology of HIVD have indicated that activation of microglia and astrocytes appears to play a pivotal role in the induction of neurophysiological impairment, both by their ability to replicate the virus actively and by the release of different soluble inflammatory mediators including cytokines, chemokines, free radicals and proteinases, such as matrix metalloproteinases (MMPs), that are toxic for myelin and neurons (Janabi et al., 1998).

MMPs are proteolytic enzymes known to degrade the components of the extracellular matrix such as collagen, elastin, fibronectin and laminin (Woessner, 1991; Shapiro, 1998). In physiological conditions, their activity is the result of a careful balance between two processes: activation of proenzymes and inhibition by tissue inhibitors of MMPs (TIMPs). Alteration of this balance may predispose to pathological events leading to inflammation and demyelination (Hartung and Kieseier, 2000).

Among MMPs, the subfamily of gelatinases seems to be involved in mechanisms of T cell migration into the CNS and blood–brain barrier (BBB) disruption in the course of HIV-associated neurological diseases (Romanic and Madri, 1994; Nottet, 1999). In particular, in vitro HIV-1 infection of T cells and monocytes increases the secretion of MMP-9 and the ability of these cells to traverse artificial basement membrane barriers (Dhawan et al., 1992; Weeks et al., 1997). It has been recently demonstrated that HIV-1 Nef protein induces BBB disruption in rats via MMP-9 induction (Sporer et al., 2000) and that HIV-1 Tat neurotoxicity is prevented by MMP inhibition (Johnston et al., 2001). We recently demonstrated that the activity of MMP-9 is increased in CSF of patients with HIV-associated neurological diseases (Liuzzi et al., 2000) and it has been recently shown that brain-derived cells, stimulated with the pro-inflammatory cytokine tumour necrosis factor (TNF)-α, are able to release MMP-9 (Conant et al., 1999). On the basis of these observations and since the release of MMPs from glial cells may represent a key event in the pathogenesis of HIV-associated demyelination, we studied the effects of two antiretroviral drugs commonly used for the treatment of HIV-infected patients on the release of MMPs from glial cell cultures. In this paper, we demonstrate that both zidovudine (AZT), a reverse transcriptase inhibitor, and indinavir (IDV), a protease inhibitor, are able to dose-dependently inhibit the release of MMP-2 and MMP-9 from astrocyte and microglia cell cultures.

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Gibco (Paisley, Scotland). Gelatin, DNase I, poly-l-lysine (PLL), trypsin, LPS, 1,10 phenanthroline (PA), Trypan blue and AZT were provided by Sigma (St. Louis, MO, USA.). IDV was from Merck Research Laboratories (Rahway, NJ, USA). Standard proteins and R-250 Coomassie Brilliant Blue were purchased from Bio-Rad (Hercules, CA, USA). Glial fibrillary acidic protein (GFAP)-specific polyclonal antisemur was from Cappel (Scarborough, Ontario, Canada). Polyclonal anti-ED 1 monocyte/macrophage antibody was purchased from Serotec (Oxford, UK). Polyclonal anti-MMP-2 and MMP-9 were from Chemicon (Temecula, CA, USA). Purified MMP-2 and anti-MMP-9 were purchased from Alexis Biochemicals (San Diego, CA, USA). The MMP-9 was derived from human neutrophil granulocytes and thus also contains covalent MMP-9-NGAL complex. Primer pairs specific for MMP-2, MMP-9 and GAPDH were from Sigma Genosys (Cambridge, UK). RNeasy mini kit was from Qiagen (Valencia, CA, USA). All the reagents for reverse transcriptase–polymerase chain reaction (RT–PCR) were purchased from Invitrogen (San Diego, CA, USA). The enhanced chemioluminescent reagent (ECL) was provided by Amersham Biosciences (Uppsala, Sweden).

**Methods**

**Preparation of microglial and astrocyte cultures**

Microglial cells were prepared from primary cell cultures of neocortical tissues from 1-day-old rats as described by Nakajima et al. (1989). Briefly, brains were cleaned of meninges and blood vessels, and the dissected neocortical tissues were minced by passage through a stainless steel mesh (40 mesh) and incubated with 0.25% trypsin and 0.01% DNase in DMEM for 10 min at 37°C. After addition of FBS, the dissociated cells were passed through a 100 mesh and viability of cells was assessed by Trypan Blue dye exclusion. Cells were plated in PLL-coated flasks (75 cm²) at a density of 1.5 × 10⁷ viable cells/flask in DMEM, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FBS and maintained at 37°C in a 5% carbon dioxide incubator with a renewal of medium twice a week. After 7–10 days in culture, the flasks were shaken for 10 min at 100 r.p.m. on a rotary shaker and the cells floating into the medium were transferred to plastic dishes and allowed to adhere at 37°C. Unattached cells were removed after 1 h by washing with DMEM. The strongly adhering cells showed a homogeneous morphology characteristic of microglia as reported by several authors (Gebicke-Haerter et al., 1989; Nakajima et al., 1989). By immunocytochemical analysis, ≥98% of the cells stained positively with anti-mouse monocyte/macrophage antibody and were negative with anti-GFAP antibodies.

For the preparation of astrocyte cultures, oligodendrocytes were separated from the astrocytes by mechanical dislodging and then the astrocytes were obtained by trypsinization (0.25% trypsin/0.02% EDTA) (McCarthy and De Vellis, 1980). Astrocytes were purified by three repetitions of replating and trypsinization to deplete cultures of microglia and oligodendrocytes. The purity of the final cell culture was assessed by immuno-staining for GFAP. More than 98% of the cells were GFAP-positive in all the preparations.

**Treatment of microglia and astrocytes with LPS, IDV and AZT**

One day after seeding at a density of 1 × 10⁵ cells/ml in 96 well-plates, the microglial cells and astrocytes were washed twice with...
serum-free DMEM, activated with LPS at the final concentrations of 1 μg/ml and simultaneously treated with AZT or IDV at the final concentrations of 50 nM, 100 nM and 500 nM. Cells were then incubated for 24 h in 100 μl of serum-free DMEM.

Negative and positive control supernatants were obtained from unstimulated and untreated microglial cells and astrocytes in 100 μl of DMEM (negative control), cells stimulated with LPS (positive control), and unstimulated cells treated with AZT or IDV at the final concentrations of 50, 100 and 500 nM. The incubations were performed in 100 μl of serum-free DMEM for 24 h at 37°C, 5% carbon dioxide. At the end of the incubation period, the medium was collected and cells were lysed in medium containing 1% Triton X-100. Culture supernatants and lysates were stored at -20°C until use.

**Detection of gelatinases**

Gelatinases in cell culture supernatants were determined by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) zymography according to a modification of the method of Heussen and Dowdle (1980) as described by Liuzzi et al. (1999). Briefly, 50 μl of culture supernatant was supplemented with 30 μl of electrophoresis-loading buffer containing SDS. The samples were then separated in a 7.5% polyacrylamide gel which had been co-polymerized with 0.1% (w/v) gelatin. Stacking gels contained 5.4% polyacrylamide. The electrophoresis was performed at 4°C for ~18 h at 80 V. After electrophoresis, the gels were washed for 2 × 20 min in 2.5% (w/v) Triton X-100/10 mM CaCl₂ in 50 mM Tris-HCl, pH 7.4 (washing buffer) in order to remove SDS, and then incubated for 24 h at 37°C in 1% (w/v) Triton X-100/50 mM Tris-HCl/10 mM CaCl₂, pH 7.4 (developing buffer).

For the development of the enzyme activity, the gels were stained with Coomassie Brilliant Blue R-250 and destained in methanol/acetonic acid/water. Gelatinase activity was detected as a white band on a blue background and was quantified by computerized image analysis through two-dimensional scanning densitometry using the Image Master 1D program (Pharmacia Biotech, Uppsala, Sweden). Gelatinase activity was expressed as optical density (OD) × mm², representing the scanning area under the curves which takes into account both brightness and width of the substrate lysis zone.

**Western blot analysis**

Equivalent supernatants were used in immunoblot analysis for the detection of MMP-2 and MMP-9 proteins. Aliquots of 50 μl were
precipitated with 1 ml cold acetone at ±20°C for 1 h. After centrifugation, dry pellets were mixed with 12 ml of SDS-buffer and electrophoresed in 10% SDS-PAGE gels. Proteins were then transferred to polyvinylidene fluoride (PVDF) and the membrane was blocked in phosphate-buffered saline (PBS) with 0.25% Tween 20 (blocking buffer) for 30 min. The blots were incubated with anti-MMP-2 and anti-MMP-9 antibodies diluted 1:1000 in blocking buffer at 4°C overnight. Blots were washed three times in PBS with 0.05% Tween 20 (washing buffer) and subsequently incubated with rabbit anti-rat peroxidase-conjugated Ab diluted 1:20,000 in washing buffer. After 1 h incubation at room temperature, the blots were washed three times and enhanced chemiluminescence reagents were used for development.

**RT–PCR**

Total cellular RNA was isolated from astrocytes and microglial cells using the Qiagen RNeasy mini kit according to the manufacturer’s instructions. RNA was ethanol-precipitated, resuspended in RNase-free water, and its quantity and quality determined spectrophotometrically at 260/280 nm. RNA samples were stored at ±70°C. Complementary DNA (cDNA) was prepared by reverse transcription using random primers. The cDNAs were used to amplify a 591 bp fragment using specific primers (sense 5'-GTC ACT CCG CTG CGC TTT TCT CG-3'; antisense 5'-GAC ACA TGG GGC ACC TTC TGA-3') for the rat MMP-2 sequence and a 541 bp fragment using specific primers (sense 5'-CGG AGC ACG GGG ACG GGT ATC 3'; antisense 5'-AAG ACG AAG GGG AAG ACC CAC ATC 3') for the rat MMP-9 sequence. Thirty cycles of PCR were performed each consisting of denaturation at 94°C, annealing at 59°C and extension at 72°C in a thermal cycler (PTC-100 Programmable Thermac Controller, MJ Research, Inc., Waltham, MA, USA). PCR products were separated electrophoretically on 1.5% agarose gels and visualized by ethidium bromide staining. Gels were then processed for densitometric analysis as described for protein gels. Standard molecular size markers, negative control (PCR mix without sample cDNA) and positive controls were run with each PCR assay. Amplification of a 308 bp fragment of rat GAPDH (sense 5'-TCC CTC AAG ATT GTC AGC AA-3'; antisense 5'-AGA TCC ACA ACG GAT ACA TT-3'), a relatively invariant internal reference RNA, was performed in parallel, and cDNA amounts were standardized to equivalent...
GAPDH mRNA levels. The primer sets specifically recognize only the genes of interest as indicated by amplification of a single band of the expected size of the PCR products.

Statistical analysis
Comparison between LPS-activated cells and cells treated with AZT or IDV after LPS activation were evaluated by Student’s t-test. Differences were considered significant if \( P < 0.05 \).

Results
AZT and IDV reduce MMP-2 and MMP-9 levels in LPS-stimulated astrocytes and microglia
Primary astrocytes and microglial cells were stimulated with LPS and simultaneously treated with different concentrations of IDV and AZT in serum-free DMEM. Cell supernatants collected after 24 h incubation were subjected to gelatin zymography for the assessment of MMP-2 and MMP-9 levels. As shown in Fig. 1A and C, astrocytes secreted only the 67 kDa MMP-2 and only traces of the 92 kDa MMP-9 in control conditions. Treatment with LPS induced significantly MMP-9 and increased the levels of MMP-2. In addition, after treatment with LPS, a band of apparent molecular mass of 72 kDa was also observed in some gels, corresponding to the pro-form of MMP-2. The in vitro treatment of astrocytes with both AZT and IDV inhibited dose-dependently the levels of both MMP-9 and MMP-2 in LPS-activated astrocytes. Quantitations of the data from three experiments with different cell populations are shown in Fig. 1B and D. The results indicate that the strongest inhibition of MMP-9 and MMP-2 was reached at the concentration of 500 nM for both AZT and IDV.

The zymographic analysis of culture medium from microglial cultures yielded completely different results in that mainly changes in MMP-9 levels were observed.

Figure 2A illustrates the zymography from an individual experiment. Under basal conditions and after 24 h incubation,
microglia released only MMP-2 and traces of MMP-9 into the culture medium. The stimulation of microglial cells with LPS consistently induced an increase of MMP-9 levels. In contrast, the treatment of microglia with AZT and IDV inhibited dose-dependently the amounts of MMP-9 produced by LPS-activated microglia, but not the levels of MMP-2 which were constitutively expressed. In general, the changes of MMP-9 levels in multiple microglial culture media after treatment with AZT and IDV are shown in Fig. 2B. The results indicated that the strongest inhibition of MMP-9 activity was reached at concentrations of 500 nM for both AZT and IDV, whereas no significant variation was observed for MMP-2.

As demonstrated by microscopical observation and cytotoxicity assay, AZT and IDV were not toxic for glial cells at the used concentrations (data not shown).

AZT and IDV do not directly inhibit the activity of purified MMP-2 and MMP-9

In an in vitro assay, we also tested the ability of IDV and AZT to inhibit the two types of purified MMPs. To do this, 0.35 μg of purified MMP-2 and 0.88 μg of purified MMP-9 were separated by gelatin zymography and the zymogram was incubated in developing buffer containing AZT or IDV at a final concentration of 500 nM. As a control, MMP-2 and MMP-9 were incubated in the presence of PA (final concentration 1 mM), an inhibitor of MMPs. The experiments showed that IDV and AZT did not exert any direct inhibition on the enzymatic activity of MMP-2 and MMP-9. On the contrary, PA treatment completely blocked the activity of both MMP-2 and MMP-9 (Fig. 3).

Inhibition of MMP-2 and MMP-9 mRNA and protein expression in astrocytes and microglia by AZT and IDV

To determine whether the inhibition of MMP-2 and MMP-9 production in LPS-stimulated microglia and astrocyte supernatants by AZT or IDV results from the inhibition of mRNA
or protein expression, RT–PCR and western blot analysis were performed. As shown in Fig. 4, in astrocytes both AZT and IDV inhibited dose-dependently the LPS-induced expression of MMP-9 as well as MMP-2 mRNA, whereas the expression of GAPDH mRNA, a housekeeping gene product used as an internal control, was unchanged. On the contrary, when microglia was subjected to RT–PCR, only the LPS-induced expression of MMP-9 mRNA was dose-dependently inhibited by both AZT and IDV while the expression of MMP-2 remained unchanged, as also observed for GAPDH (Fig. 5). Results obtained by western blot analysis are shown in Fig. 6. When blots from the astrocyte-conditioned media were probed with a specific antibody raised against MMP-9, a 92 kDa immunoreactive band, corresponding to MMP-9, was observed. In particular, the LPS-induced increase in MMP-9 immunoreactivity was inhibited by both AZT and IDV in a dose-dependent manner. Similar results were observed when conditioned media from microglia were probed with the MMP-9 antibody. The western blot analysis of conditioned media from astrocytes and microglia probed with specific antibodies raised against MMP-2 gave results which were consistent with the data observed by zymography and RT-PCR (data not shown).

**Discussion**

In this paper, we studied the effects induced by the antiretroviral drugs AZT and IDV on the synthesis and the release of MMPs by glial cells and we show evidence of the ability of these drugs to inhibit the activity and the expression of MMP-2 and MMP-9 in astrocyte and microglial cell cultures.

Several antiretroviral drug combinations are currently in use for the treatments of HIV-1 infected patients. These can significantly prolong the survival of patients through delay of disease progression, suppression of HIV replication and improvement of immunological functions. In particular, the frequency of HIV-1-related CNS diseases has been reduced in part through the reduction of both viral load in blood and continuous penetration of virus into the brain (Enting et al., 1991; Liuzzi et al., 1992). In the present study, we demonstrated that both AZT and IDV are able to dose-dependently inhibit the expression of MMP-2 and MMP-9 in astrocyte and microglia cells. We did not find synergy between both drugs in the inhibition of MMPs (data not shown).

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To test the effect of antiretroviral therapy on MMPs, we activated glial cells with LPS, a potent inducer of MMP-9 in microglia and of MMP-2 and MMP-9 in astrocytes cells (Gottschall and Yu, 1995a,b). Simultaneously, we treated cells with two different types of antiretroviral drugs: AZT, an inhibitor of the reverse transcriptase of HIV and IDV, an inhibitor of HIV protease. Both of these possess the ability to traverse the BBB and may reach glial cells in vivo (Burger et al., 1993; Stahle et al., 1997; Martin et al., 1999). In particular, AZT has proved effective in the treatment of HIV-associated dementia and we demonstrated the ability of this drug to reduce the release of MBP from myelin sheath (Mastroianni et al., 1992). In the present study, we demonstrated that both AZT and IDV are able to dose-dependently inhibit the expression of MMP-2 and MMP-9 from LPS-activated astrocytes and of MMP-9 from LPS-activated microglia without any toxic effect on glial cells. Given that for the treatment of HIV patients a combination of drugs is used, we also tested the combined effect of AZT and IDV on astrocytes and microglia cells. We did not find synergy between both drugs in the inhibition of MMPs (data not shown).
When AZT and IDV were directly incubated with purified MMPs, no inhibition of enzymatic activity was detected. This finding suggests that the inhibitory effect seen on MMPs could be ascribed to a modulation of the expression of the enzymes induced by drugs. This hypothesis is also supported by our observation that, in microglial cells, in which only the activity of MMP-9 is regulated while MMP-2 is constitutively expressed (Liuzzi et al., 1999), the inhibitory effect of AZT and IDV was found only on MMP-9 and not on MMP-2. In addition, we performed a set of experiments using western blot and PCR analysis to detect protein and mRNA expression of MMP-2 and MMP-9. The results showed that AZT and IDV exert an inhibitory effect only on the expression of MMP-9 in microglia, and of MMP-2 and MMP-9 in astrocytes. The inhibitory effects of AZT and IDV on MMP expression at both protein and mRNA levels probably result from attenuated transcription. Mechanisms by which AZT and IDV inhibit gene transcription are still under investigation.

Overall, these findings indicate that AZT and IDV down-regulate MMP-9 and MMP-2 levels by inhibiting the expression of MMPs mRNA and protein in rat primary astrocyte and microglial cells.

Preliminary results obtained in our laboratory indicated also that the expression of TIMP-1 is reduced in LPS-activated astrocytes after treatment with AZT or IDV (data not shown). Recent studies indicated that the activation of glial cells with proinflammatory cytokines and LPS increases the expression of TIMP-1, the inducible type of TIMP, probably as a regulatory mechanism to control activation of MMPs at sites of inflammation and tissue damage (Gomez et al., 1997; Suryadevara et al., 2003). Thus, the reduced expression of TIMP-1 after treatment with AZT or IDV might represent a compensatory mechanism to control the exaggerated production of TIMPs that could lead to fibrotic processes (Mastroianni et al., 2002). However, how the inhibition of MMP and TIMP-1 expression by AZT or IDV might affect in vivo the ratio MMP/TIMP remains to be established.

The introduction of potent antiretroviral drugs in clinical practice has greatly changed the natural history of HIV infection with significant reduction in the morbidity and mortality. The main clinical and immunological benefits induced by HIV drugs derive from the ability of these drugs to block the HIV reverse transcriptase and HIV protease with subsequent suppression of viral replication. Nevertheless, these compounds provide substantial benefits, even in patients with persistent viral replication, suggesting an effect that is independent from the antiviral activity (Kaufmann et al., 1998; Levitz, 1998; Mezzaroma et al., 1999). Such an effect may be the modulation of MMPs.

In addition, our findings may have clinical implications beyond the treatment of HIV-infected patients, for instance in pathological processes that involve abnormal MMP production. An involvement of MMP in the onset of different diseases, including tumour growth and metastasis, rheumatoid arthritis, cardiovascular diseases as well as neurological disorders such as Alzheimer’s, multiple sclerosis and glioma has already been demonstrated (Opdenakker and Van Damme, 1994; Yong et al., 1998; Kieseier et al., 1999). All these diseases involve abnormal MMP expression pointing to MMPs as pharmacological targets. In this context, the use in the 1990s of synthetic MMP inhibitors to treat cancer and other diseases was introduced and several MMP inhibitors have entered clinical trials as a result of data from animal models (Whittaker et al., 1999). However, the results from these clinical trials have been equivocal, with some studies being terminated because of lack of efficacy or safety concerns (Brown, 2000; Pavlaki and Zucker, 2003). As a contrast, the antiretroviral drugs AZT and IDV have been successfully and safely used for many years for the treatment of AIDS. Recent experimental findings open new therapeutic perspectives for these drugs, especially for HIV protease inhibitors, which have been found to possess anti-inflammatory, anti-angiogenic and anti-tumour activity (Monini et al., 2003). In addition, these drugs have a good penetration into the CNS, a prerequisite for the possible management of neurological diseases. This property may be particularly relevant in diseases such as multiple sclerosis, in which the intrathecal synthesis of MMP-9 has been demonstrated (Liuzzi et al., 2002). On these grounds, the results of our study open the possibility to use antiretroviral drugs, more likely HIV protease inhibitors, as potential candidates for the experimental treatment of neurological disorders in which the inhibition of MMP expression could have clinical benefits.

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