Monocytoid Differentiation of Freshly Isolated Human Myeloid Leukemia Cells and HL-60 Cells Induced by the Glutamine Antagonist Acivicin

By Kim E. Nichols, Shobha R. Chitneni, Joseph O. Moore, and J. Brice Weinberg

Previously we showed that starvation of HL-60 promyelocytic leukemia cells for a single essential amino acid induced irreversible differentiation into more mature monocytic-like cells. Although not an essential amino acid, glutamine is important in the growth of normal and neoplastic cells. The glutamine analogue, αS5S-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin) inhibits several glutamine-utilizing enzymes and therefore depletes cells of certain metabolic end products. The current study was designed to examine in vitro the effects of acivicin on growth and differentiation of several established human myeloid leukemia cell lines, including the HL-60 cell line, and of freshly isolated cells from patients with acute nonlymphocytic leukemia (ANLL). Four-day culture of HL-60 cells with acivicin at concentrations of 0.1 to 10.0 μg/mL (0.56 to 56 nmol/L) decreased cell growth by 33% to 98% as compared with untreated control cells. Viability of cells was ≥92% for untreated cells and 93% to 41% for acivicin-treated cells. Cells treated with acivicin differentiated along a monocytic pathway as shown by increased H2O2 production and α-naphthyl butyrate esterase (NSE).

The HL-60 promyelocytic leukemia cell line is useful in examining the process of differentiation. This cell line is remarkable for its ability to differentiate along either a monocytic or myeloid pathway under appropriate culture conditions. In previous studies, we showed that starvation of HL-60 cells for a single essential amino acid inhibits proliferation and induces irreversible monocytic differentiation. Glutamine, though not an essential amino acid, is critical for the growth of normal and neoplastic cells. Several of glutamine’s roles include participation in protein, purine, and pyrimidine synthesis, and as an energy source in mammalian cell metabolism. The glutamine analogue acivicin has antitumor effects secondary to its function as a glutamine antagonist. In vitro cultures of mammalian cells with acivicin demonstrate that acivicin inhibits several glutamine-dependent amidotransferases, especially those important in synthesis of cytidine triphosphate and guanosine triphosphate, resulting in depletion of intracellular pools of these nucleotides.

In previous in vitro studies, acivicin has shown antitumor activity against murine L1210 leukemia cells, and in vivo experiments it inhibits growth of human breast and lung tumor xenografts in athymic mice. Early phase I and II trials using acivicin have shown some benefit in the treatment of several solid tumor types (eg, colon, breast, and ovarian carcinomas, and melanoma). In a phase I-II trial of acivicin, two of six adult patients with relapsed or refractory acute leukemia had initial cytotreduction of leukemia cells, but no partial or complete remissions were induced. The purposes of our investigations were several-fold: to examine the effects of acivicin on growth and differentiation of the HL-60 promyelocytic leukemia cell line and other myeloid leukemia cell lines, and to assess whether acivicin has in vitro antiproliferative and differentiating activity against freshly isolated peripheral blood leukemia cells from ANLL patients.

Results show that acivicin effectively decreases the growth and viability of HL-60 U937, K562, and KG-1 myeloid leukemia cell lines. In addition, acivicin induces differentiation of HL-60 cells along a monocytic pathway, but it did not consistently differentiate the other cell lines studied. Acivicin decreases cell survival and induces differentiation of freshly isolated cells from ANLL patients, suggesting that it may be beneficial as a therapeutic agent for ANLL. In parallel experiments, acivicin had variable effects on proliferation and function of mononuclear cells from normal controls.

MATERIALS AND METHODS

Cell lines. The HL-60, U937, K562, and KG-1 myeloid leukemia cell lines were obtained from the American Type Culture collection (Rockville, MD). All cells were routinely passed at a concentration of 1.0 to 2.5 × 10⁶ cells/ml in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sterile Systems, Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin. For HL-60 cells, cell passage number was <60, and <1% to 2% of cells were positive.
for α-naphthyl butyrate esterase (NSE) at the start of all experiments.

Culture conditions. For experiments, cells were recovered from routine growth medium by centrifugation, washed twice with phosphate-buffered saline (PBS), and resuspended at a density of 2.5 × 10⁶ cells/ml in RPMI 1640 supplemented as in routine cultures. Acicin, diluted in distilled water at a stock concentration of 4 mg/mL, was added in appropriate amounts at initiation of all cultures. Cultures were maintained at 37°C with 100% humidity and 5% CO₂ for one to seven days. Before all assays, cells were harvested by centrifugation and washed three times with PBS, and cell number and viability were determined by exclusion of 0.1% trypan blue (in PBS).

Isolation and culture of peripheral blood leukocytes (PBLs). PBLs were isolated from normal controls and from ANLL patients (with appropriate consent according to protocols approved by the Duke and VA Institutional Review Boards) by Ficoll-Hypaque gradient sedimentation as previously described. After centrifugation, cells were washed three times with PBS and assessed for cell number and viability. When >50% were blasts, the cells were cultured in RPMI 1640 supplemented with 10% FBS and penicillin/streptomycin as described. Culture conditions were identical to those described for the leukemia cell lines except that in these experiments, final acicin concentrations were 0, 1.0, 10, and 100 μg/mL.

Purification of peripheral blood monocytes, lymphocytes, and peritoneal macrophages. For certain experiments, mononuclear cells were isolated into populations of lymphocytes and monocytes by Percoll gradient sedimentation as previously described. After Percoll sedimentation, cells were washed three times with PBS and were assessed for cell number and viability. Peritoneal macrophages were isolated from peritoneal fluid by Ficoll-Hypaque gradient sedimentation as previously described. Before culture, cells were washed twice with medium, allowed to adhere to microtiter plates in 200 μl medium including all additives. Then either 1 μCi (methyl-3)thymidine (2 Ci/mmol, NEN, Boston), (5,6-3)Hurtidine (2 Ci/mmol, Amersham, Arlington Heights, IL), or L-(4,5-3)leucine (57 Ci/mmol, Amersham) was added to each well. After 18, 18, or four hours of culture, respectively, cells were harvested onto glass fiber filter discs with a multiple automated sample harvester (Belco, Vineland, NJ) and filter discs were analyzed for radiation content as described previously.

Materials. Purified recombinant interferon-γ (IFN-γ) was obtained from Genentech (San Francisco), and purified TNF was obtained from Cetus (Emeryville, CA). Acicin was from Upjohn (Kalamazoo, MI). All other reagents, unless otherwise specified, were purchased from Sigma.

RESULTS

Cell growth. When grown in the presence of acicin, HL-60 cells show a decrease in proliferation and viability which occurs in a time- and dose-dependent fashion. As shown in Fig 1, this decrease in proliferation is gradual, with maximal effects at days 4 to 5. In general, after four-day culture with acicin (0.1, 1.0, 10 μg/mL), cell number recovered was 67%, 23%, and 12% of control cell number (with control cells having increased 150% to 250% from initial seed during this culture period). Viability of cells was >92% for control cells and 93%, 69%, and 41% for acicin-treated cells.

Cell morphology and cytochemistry. Examination of Wright's-stained cytopsin preparations of HL-60 cells cultured with acicin showed cells that were larger and more vacuolated and that had more ruffled membranes than control cells. These changes were comparable to those observed in cells cultured in amino acid-deficient medium that we have described previously. The change in morphology suggested monocyctic differentiation; however, these cells were not adherent to the plastic culture dishes as are more mature monocytes or HL-60 cells induced to differentiate.

Fig 1. Growth curves of HL-60 cells in medium containing different concentrations of acicin. Viable cell number is expressed as total viable cells per culture dish. Results are from one experiment representative of five experiments.
into monocytes by phorbol diesters.\textsuperscript{18} When we assessed for NSE content, which is useful as a marker of monocytic differentiation,\textsuperscript{19} we found that culture with acivicin resulted in increased expression of NSE. As shown in Fig 2, acivicin-induced NSE content was dose dependent, with 10 \( \mu \)g/mL acivicin inducing maximal NSE content. Parallel experiments showed that the increase in NSE content was time dependent. When analyzed after four-day culture with 0, 0.1, 1.0, and 10 \( \mu \)g/mL acivicin, 2\%, 6\%, 42\%, and 56\% of cells were positive for NSE. To determine the reversibility of the differentiation, cells were cultured for four days with acivicin, washed twice in PBS to remove the drug, and recaptured for an additional five days. NSE content was unchanged, suggesting that this process was irreversible (data not shown).

\textbf{Peroxide production.} Differentiation of HL-60 cells into more mature myeloid or monocytic cells is characterized by acquisition of the ability to produce reactive oxygen species.\textsuperscript{2} As with NSE, acivicin-treated cells showed an increase in \( \text{H}_2\text{O}_2 \) production that was comparable in time and dose dependence (Fig 2) and irreversibility. At day 4 of culture, cells grown in the presence of 0, 0.1, 1, and 10 \( \mu \)g/mL acivicin produced 13.8, 112, and 264 nmol/h/\( 10^6 \) cells \( \text{H}_2\text{O}_2 \) when stimulated with phorbol myristate acetate (PMA).

\textbf{Reversibility of differentiation by addition of nucleo-}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Dose response (A) and time course (B) for NSE induction in acivicin-treated cells and dose response (C) and time course (D) for \( \text{H}_2\text{O}_2 \) production in acivicin-treated cells. Cells were cultured with varying concentrations of acivicin for four days (A or C), or with 10 \( \mu \)g/mL acivicin for different periods of time (B and D), and then were harvested and assayed for NSE content and \( \text{H}_2\text{O}_2 \) production. Values are the mean ± SD of eight experiments.}
\end{figure}
side. To determine whether differentiation could be prevented by adding exogenous nucleosides, thereby bypassing acivicin-induced metabolic blocks, cells were cultured with acivicin at a concentration of 5 μg/mL with one or more of the nucleosides shown in Table 1. Acivicin 5 μg/mL was chosen because it was sufficient to produce appreciable differentiation after four-day culture. As shown in Table 1, addition of large concentrations (10 mmol/L) of cytidine and guanosine could prevent acivicin-induced decrease in cell viability with little effect on acivicin-induced decrease in cell number. The combination of cytidine and guanosine could also partially prevent differentiation in terms of H2O2 production and NSE content. Culture with adenosine or hypoxanthine with acivicin enhanced the ability of the cells to produce H2O2.

Expression of the chemotactic peptide receptor. We showed previously that IFN-γ, tumor necrosis factor (TNF), or dimethylsulfoxide (DMSO)-induced differentiation of HL-60 cells is characterized by acquisition of FMLP-R. In addition, expression of this receptor is associated with the ability of the cells to polymerize actin, which is believed to be important in the processes of chemotaxis and phagocytosis. Acivicin-treated cells demonstrated an increase in the percentage of cells positive for FMLP-R as well as an increase in mean fluorescence intensity (density of receptor expressed/cell) (Fig 3).

![Fig 3](image-url)
Measurement of DNA, RNA, and protein synthesis. To characterize further the events involved in differentiation of HL-60 cells after incubation with acivicin, we measured the level of DNA, RNA, and protein synthesis in control and acivicin-treated cells. Cells cultured with 0.1, 1.0, and 10 μg/mL acivicin had DNA synthesis that was 85%, 20%, and 12% of control untreated cells. RNA synthesis was 86%, 66%, and 9% of control. Protein synthesis also decreased to 47%, 26%, and 19% of control.

Cooperative effects of acivicin and other differentiating agents. TNF, IFN-γ, dihydroxyvitamin D₃ (D₃), and phorbol diesters induce maturation of HL-60 cells into monocyte/macrophage-like cells. In contrast, DMSO, retinoic acid (RA), and dibutyryl CAMP induce maturation of HL-60 cells along a myeloid pathway. To determine whether culture with acivicin would have any effects on differentiation in the presence of other inducing agents, cells were either co-cultured with acivicin (5 μg/mL) and the inducer for four days or were preincubated with acivicin (5 μg/mL) for four days, washed free of acivicin, and recaptured for an additional four or five days with an inducer, and then were analyzed for differentiation. As shown in Fig 4, acivicin augmented H₂O₂ production induced by IFN-γ, TNF, D₃, RA, and DMSO by 5.3-, 3.1-, 6.3-, 2.2-, and 4.4-fold, respectively. Acivicin augmented NSE content induced by IFN-γ, TNF, and D₃ by 3.3-, 5.5-, and 4.1-fold.
respectively. When the cells were cultured with acivicin and DMSO or RA, two agents which induce neutrophilic differentiation of HL-60 cells, there was no enhancement of the relatively monocytic leukemia cell lines, cells from the KG-1, K562, and K562 cell lines\textsuperscript{33} were cultured with acivicin and assayed for cell number, viability, H$_2$O$_2$ production, and NSE content. For K562, maturation along the erythrocytic pathway was examined by change for examination of intracellular hemoglobin content as previously described.\textsuperscript{34} As shown in Table 2, culture with acivicin resulted in decreased cell proliferation and viability for all cell lines examined. However, culture with acivicin did not consistently lead to differentiation of these cell lines.

**Differentiation of ANLL patient samples.** To examine the effects of acivicin on the survival and differentiation of freshly isolated ANLL cells, cells were incubated for four days with 0, 1, 10, and 100 $\mu$g/mL acivicin and then harvested and assessed for cell number, viability, morphology, H$_2$O$_2$ production and NSE content. Results are summarized in Fig 5A through D. At these doses, acivicin decreased cell survival in ten of ten cases with mean reductions in cell number of 39%, 49%, and 54%, respectively, over control cells. Cell viability also decreased in ten of ten cases with reductions (Fig 5B). Mean PMA-induced H$_2$O$_2$ production by ANLL cells was 72, 130, 120, and 150 nmol/h/10 x 10$^6$ cells for control and acivicin-treated cells (1, 10, and 100 $\mu$g/mL), with enhancement of H$_2$O$_2$ production in six of ten cases. At the same doses of acivicin, NSE content increased by 2.4-, 3.0-, and 3.2-fold over control cells, with increased NSE content in five of ten cases. Despite changes in H$_2$O$_2$ production and NSE content, morphologic changes on Wright's-stained cells consistent with differentiation or maturation were not always observed.

**Effects of acivicin on normal PBLS and peritoneal macrophages.** Parallel experiments were performed to determine the effects of acivicin on proliferation and function of either peripheral blood lymphocytes or peripheral blood monocytes/peritoneal macrophages. These experiments demonstrated that at concentrations of 0.1, 1.0, and 10 $\mu$g/mL acivicin inhibited phytohemagglutinin-induced proliferation\textsuperscript{35} of normal lymphocytes by 3%, 90%, and 99% over untreated cells. Culture with acivicin at these doses had no effect on H$_2$O$_2$ generation by either peripheral blood monocytes or peritoneal macrophages (data not shown).

**DISCUSSION**

Because of the integral role that glutamine plays in intermediary metabolism of neoplastic cells, glutamine analogues, antimetabolites, and glutamine-depleting enzymes have been the focus of chemotherapy regimens for several years.\textsuperscript{4} Glutamine is important in the production of several amino acids, nucleotides, and complex polysaccharides. In neoplastic or transformed cells, glutamine is particularly important in glutamine-dependent synthesis of purines and pyrimidines and is therefore closely linked to DNA synthesis and cell replication. Among the enzymes of significance are phosphoribosylpyrophosphate amidotransferase, carbamoylphosphate synthetase, GMP synthetase, and CTP synthetase, all of which are rate-limiting enzymes for either purine or pyrimidine synthesis and, more specifically, for synthesis of CTP or GTP.\textsuperscript{36,37} As demonstrated by Weber, many of the enzymes involved in purine and pyrimidine metabolism have increased activity in a large number of animal and human tumor cells. The degree of increased activity correlates well with tumor cell growth rate, and the common pattern of enzymic changes is believed to reflect a pattern of gene expression which confers selective reproductive advantage.\textsuperscript{38} In association with these changes, intracellular pools of glutamine are depleted in tumor cells as compared with normal controls. Further depletion of the glutamine pool by glutamine antimetabolites or glutamine-degrading enzymes is believed to provide a strategic approach in chemotherapy.

A wide variety of glutamine analogues is available for investigation, including azaserine, 6-diazo-5-oxo-L-norleucine, and acivicin. Our studies were designed to examine in vitro the effects of acivicin on growth and differentiation of the promyelocytic leukemia cell line HL-60 and on survival and differentiation of freshly isolated ANLL cells. We have

**Table 2. Effects of Acivicin on Growth and Differentiation of Other Myeloid Leukemia Cell Lines**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Acivicin ($\mu$g/mL)</th>
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<tr>
<td></td>
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</tr>
<tr>
<td>Cell number (millions)</td>
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<td>K562</td>
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</tr>
<tr>
<td>KG-1</td>
<td>33</td>
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<td>Viability (viable, %)</td>
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</tr>
<tr>
<td>U937</td>
<td>99</td>
</tr>
<tr>
<td>K562</td>
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<tr>
<td>KG-1</td>
<td>98</td>
</tr>
<tr>
<td>H$_2$O$_2$ (nmol/h)</td>
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</tr>
<tr>
<td>U937</td>
<td>0</td>
</tr>
<tr>
<td>K562</td>
<td>0</td>
</tr>
<tr>
<td>KG-1</td>
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<tr>
<td>NSE (cells, % positive)</td>
<td></td>
</tr>
<tr>
<td>U937</td>
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</tr>
<tr>
<td>K562</td>
<td>NA</td>
</tr>
<tr>
<td>KG-1</td>
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<tr>
<td>Benzidine (cells, % positive)</td>
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<tr>
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<td>K562</td>
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<tr>
<td>KG-1</td>
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Abbreviation: NA, not applicable.

Acivicin decreases growth and viability of other myeloid leukemia cell lines, but does not lead to changes in H$_2$O$_2$ production or NSE content. Cells (10 x 10$^6$) were cultured with acivicin for four days and then were harvested and assayed for changes in growth and differentiation. Results are from one experiment per cell line representative of three experiments for U937 and two experiments each for K562 or KG-1.
shown that acivicin inhibits growth of HL-60 cells and increases cell death. This agrees with the work of other investigators using other malignant cells. With comparable concentrations of acivicin, Zhen et al demonstrated growth inhibition of the rat hepatoma cell line 3924A.29 Dranoff et al showed decreased proliferation of several human glioblastoma and medulloblastoma cell lines with acivicin.40 Fischer et al also inhibited growth of the human colon carcinoma cell line, VACO5.41 In addition to growth inhibition, we demonstrated that acivicin induces morphologic and functional differentiation of HL-60 cells and freshly isolated ANLL cells into monocytelike cells capable of increased H2O2 production and characterized by increased NSE content and increased expression of FMLP-R. As with many other

Fig 5. Effect of acivicin on growth and differentiation of ANLL cells. Freshly isolated ANLL cells were cultured with different doses of acivicin for four days and then were harvested and assayed for cell growth (A), viability (B), H2O2 production (C), and NSE content (D). Values are either from ten experiments (A through C) or six experiments (D).
previously described differentiating agents, differentiation is gradual, requiring four to seven days of culture, is time and dose dependent and irreversible. We demonstrated comparable effects of 6-diabzo-5-oxo-l-norleucine on HL-60 cells (data not shown).

Acivicin-induced differentiation is accompanied by decreased DNA, RNA, and protein synthesis. Our experiments, however, did not address whether decreased DNA, RNA, and protein synthesis are a direct metabolic effect of acivicin or are secondary to terminal differentiation of cells. These findings are similar to those of other investigators who have differentiated HL-60 cells with other inducers. The broad spectrum of enzymes and cellular processes affected by this drug make it an attractive candidate as an antileukemia agent.

Understanding of the molecular events involved in differentiation of HL-60 cells by conventional differentiating agents or by newer agents such as acivicin is incomplete. Some researchers have proposed that alterations in DNA synthesis with subsequent alteration in gene product expression may be important. Yen et al showed that PMA-induced monocytic differentiation of HL-60 cells is enhanced by interruption of DNA synthesis by hydroxyurea. Although not well understood, inhibition of DNA synthesis during differentiation may be associated with alterations of protooncogene or growth/differentiation factor expression or both. McCachren et al showed that during dibutyryl-cAMP–induced differentiation of HL-60 cells, there is interruption of transcription and reduced expression of the c-myc oncogene.

Other investigators have suggested that alterations in intracellular pools of CDP and GTP are closely linked to the terminal differentiation of HL-60 cells. Wright et al showed that culture of HL-60 cells with tiazofuran, an inhibitor of IMP dehydrogenase, results in decreased synthesis of GTP as well as in terminal differentiation. Because acivicin also decreases cellular pools of guanosine nucleotides, this may reflect one of its roles during differentiation. In our experiments, differentiation was partially prevented by addition of exogenous guanosine and cytidine, and not by either nucleoside alone or by addition of other nucleosides. This has been demonstrated in other studies and suggests that the pyrimidine CTP is also important in the process of differentiation.

Increased cytotoxic effects of acivicin and prevented reversal of differentiation by addition of cytidine and guanosine (data not shown).

In our studies, we also show that acivicin has antiproliferative effects against the U937, K562, and KG-1 myeloid leukemia cell lines and antileukemia and differentiating effects on freshly isolated peripheral blood ANLL cells. Parallel experiments demonstrated that acivicin has potent effects on inhibiting phytohemagglutinin-stimulated lymphocyte proliferation, but did not affect monocyte or macrophage H₂O₂ production.

Because acivicin causes inhibition of neoplastic cell replication by decreasing CTP and GTP synthesis, synergism might be expected with other agents that block DNA synthesis. Weber et al showed synergistic action of acivicin with actinomycin D as measured by growth inhibition of the rat hepatoma cell line 3924A and by a decrease in clonogenic units. Use of acivicin in combination with Acinetobacter t-glutaminase-l-asparaginase by Holenberg et al showed increased antiproliferative effects in vivo against murine L1210 leukemia, murine Erlich ascites tumor cells, and murine P388 tumor cells.

Other studies investigating combinations of acivicin and purine antimetabolites (6TG), pyrimidine antimetabolites (N-phosphonacetyl-L-aspartic acid [PALA], 5-fluorouracil, or cytosine arabinoside) and the salvage pathway inhibitor dipyridamole have all shown additive or synergistic effects. Our experiments using acivicin in conjunction with the differentiating agents TNF, IFN-γ, and Δ1 showed additive effects and suggest other possible combinations with antileukemia and differentiating effects.

Our data suggest that the glutamine analogue acivicin may be important in inducing differentiation of myeloid leukemia cells. Further research with this agent may provide information concerning the molecular events involved in growth and differentiation of normal and neoplastic cells. Clinically, this agent may prove useful either alone or in combination with other agents in treating ANLL patients, with the ultimate goals of treatment being to decrease leukemia cell proliferation and to induce differentiation of leukemia cells into more mature, functional monocyte-like cells.

ACKNOWLEDGMENT

We thank Elissa Doty and Nick Mason for excellent technical assistance, and Drs Donald Granger, Henry Friedman, and Donald Trump for helpful discussions.

REFERENCES


Monocytoid differentiation of freshly isolated human myeloid leukemia cells and HL-60 cells induced by the glutamine antagonist acivicin

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