Interferon Induction by Viruses. XVI. 2-Aminopurine Blocks Selectively and Reversibly an Early Stage in Interferon Induction

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SUMMARY

A purine analogue, 2-aminopurine, reported to act as an inhibitor of protein kinase, selectively, reversibly and in a dose-dependent manner blocked a very early stage in interferon induction. With chick embryo cells and mouse L cells as hosts, and different viral inducers of interferon, maximal effects of 2-aminopurine were observed during the first 4 h of induction. At 10 mM-2-aminopurine there was a 20-fold reduction in the yield of interferon from both cell types. 2-Aminopurine and actinomycin D both prevented interferon induction with the same time course, indicating a transcriptional block to induction; however, only the action of the former was reversed upon removal of the drug. Addition of 2-aminopurine to an agarose overlay resulted in high efficiency plaque formation by vesicular stomatitis virus New Jersey (Hazelhurst) under conditions where endogenous induction of interferon and its feedback action on aged chick embryo cells normally prevented plaque formation. Two other inducible systems, representing genes involved in interferon action (both its development and activation), and those of heat shock, were not affected by 2-aminopurine. A model is presented implicating the interferon-inducible dsRNA-dependent protein kinase as an interferon induction receptor which, on interaction with dsRNA, generates an amplified signal via phosphorylation that ultimately derepresses the interferon gene(s).

INTRODUCTION

Double-stranded RNA appears to be the molecular species most likely to be responsible for initiating interferon (IFN) induction in many virus–cell systems (for reviews, see Marcus, 1983, 1984). In the most sensitive system a single molecule of dsRNA properly introduced into the cell constitutes the threshold for induction and results in a full quantum yield of IFN (Marcus & Sekellick, 1977; Marcus, 1984). Studies with NH₄Cl, a lysosomotropic agent, show that for Sindbis virus and vesicular stomatitis virus (VSV) the IFN inducer molecule, or its precursor, rapidly gains entry into the cytosol via low pH-dependent fusion from endosomal or lysosomal vacuoles (Svitlik & Marcus, 1984). Following virus attachment in the cold (Silverstein & Marcus, 1964), the IFN-inducing particle activity of virus stocks registers within a few minutes at 37 to 40 °C (Svitlik & Marcus, 1984). Once viral dsRNA reaches the cytosol as a preformed or newly synthesized molecule, cellular recognition of the dsRNA as an IFN inducer is rapid, since transcription of the IFN genes may start within 1 h (Stewart, 1981; Enoch et al., 1986). Transcription of the IFN gene(s) appears to involve removal of specific repressor molecules (Zinn & Maniatis, 1986) and initiation of transcription by an endogenous, IFN-inducible, trans-activating factor (Enoch et al., 1986; Carver et al., 1968). However, little is known regarding the nature of cellular molecules presumed to recognize and interact with dsRNA during the very early stages of IFN induction, prior to gene transcription.

In this context, Colby & Chamberlin (1969) proposed the existence of a cellular receptor protein capable of recognizing the paramount distinguishing features of dsRNA, namely its helicity and the 2′-hydroxyl groups on the sugar moieties. This concept was strengthened by the
finding that antibody to dsRNA recognizes the same gross secondary structure common to inducers of IFN (Johnston et al., 1975), and is now accepted generally (Minks et al., 1979; Torrence & DeClercq, 1984). The concept was elaborated on recently with a model in which candidate molecules for a postulated interferon inducer receptor exist in the form of cellular proteins with high affinity binding for dsRNA (Marcus, 1984). The best defined of this class of molecules, some of which are inducible by IFN, are the dsRNA-dependent 2'-5' oligo(A) synthetase and protein kinase (Gupta et al., 1979; Penn & Williams, 1985; Lengyel, 1982).

Because a single molecule of dsRNA per cell suffices to induce a quantum yield of IFN (Marcus & Sekellick, 1977), some amplification of the cellular signal triggered by dsRNA seems necessary to achieve IFN induction. Conceivably, signal amplification could result from activation by viral dsRNA of the IFN-inducible, dsRNA-dependent protein kinase (PK$_{IFN-dsRNA}$), and its ensuing phosphorylating capacity (Lasky et al., 1982; Hovanessian et al., 1986). Furthermore, IFN induction and activation of PK$_{IFN-dsRNA}$ display similar structural requirements: both reactions require an uninterrupted run of 6 to 12 base pairs and a minimal length of about 50 base pairs (DeClercq, 1974; Greene et al., 1978; Minks et al., 1979).

In this communication we report the effect on IFN induction of 2-aminopurine (2-AP), a drug that inhibits phosphorylation of eIF2-$\alpha$ and a ribosome-associated protein, P$_1$, by inhibiting protein kinase, thus allowing the activity of endogenous phosphoprotein phosphatases to dominate (Farrell et al., 1977; DeBenedetti & Baglioni, 1983). We treated cells with 2-AP to reduce the level of phosphorylation in the intact cell, and measured the cell's capacity to induce IFN and to respond to the action of IFN and heat shock. 2-Aminopurine manifested a selective, reversible and dose-dependent inhibition of an early stage in IFN induction by viruses. These results provide evidence that phosphorylation may play a critical role in the regulation of a very early step in IFN induction.

**METHODS**

**Cells and media.** Monolayers of primary chick embryo cells were prepared from 10-day-old Utility grade embryonated eggs (Spafas, Norwich, Conn., U.S.A.) and seeded in NCI medium containing 6% calf serum, and aged in vitro as described (Sekellick & Marcus, 1986). Mouse L(Y) cells were grown in MEM containing 4% calf serum. The origin and characteristics of this line relative to IFN induction and action have been described (Marcus et al., 1981).

**Viruses: source, preparation and assay.** The origin, growth and plaque assay of VSV-Indiana (HR) wild-type and VSV-T1026RI have been described (Sekellick & Marcus, 1979). VSV-New Jersey (Hazelhurst) was a gift from R. A. Lazzarini (NIH, Bethesda, Md., U.S.A.). The origin, preparation and characteristics of u.v.-irradiated avian reovirus as an IFN-inducing particle have been described (Winship & Marcus, 1980).

**Interferon induction and assay.** Detailed protocols have been described for the induction and assay of IFN in aged primary chick embryo cells (Sekellick & Marcus, 1986) and mouse L(Y) cells (Marcus et al., 1981). Chick embryo cells aged in vitro for 7 to 10 days produce very high yields of IFN (Sekellick & Marcus, 1985, 1986).

**Reagent.** Stock solutions of 150 mM-2-AP (Sigma) were prepared by dissolving the drug in glacial acetic acid diluted 1:200 in phosphate-buffered saline (PBS). Solubilization was aided by heating to 60 °C and shaking periodically with a vortex mixer. All experiments utilizing 2-AP were controlled by treating one set of cells with the appropriate amount of acetic acid-PBS diluent. This latter treatment produced the same results as those obtained from untreated cells.

**RESULTS**

2-Aminopurine inhibits interferon induction

The basic observation is illustrated in Fig. 1. When confluent monolayers of mouse L cells, or aged chick embryo cells, were infected with IFN-inducing particles of VSV or u.v.-irradiated avian reovirus, respectively, and incubated continuously with low doses of 2-AP, they produced a modest increase (about twofold) in IFN yield in four out of five experiments. At concentrations of 2-AP in excess of about 3 mM there was a dose-dependent inhibition of IFN induction. An approximately 10-fold reduction of IFN yield was observed in both cell types when the medium contained 10 mM-2-AP.

How long does it take for 2-aminopurine to achieve maximal inhibition of IFN induction?

Fig. 2 illustrates the time course of inhibition of IFN yield by 2-aminopurine. If 2-AP was added to chick embryo cells immediately after infection with u.v.-inactivated avian reovirus as
2-Aminopurine blocks interferon induction

Fig. 1. 2-Aminopurine concentration-dependent inhibition of IFN induction. Confluent monolayers containing $2 \times 10^6$ mouse L(Y) cells (a), or $2 \times 10^7$ chick embryo cells (b) in 50 mm dishes were infected (induced) with VSV-T1026R1, or u.v.-inactivated avian reovirus, each at an m.o.i. of 5, respectively, and fed medium containing different concentrations of 2-AP. The medium was harvested after 24 h at 37.5 °C (a) or 40.5 °C (b) and assayed for its IFN content. Monolayers of control mouse L(Y) cells and chick embryo cells produced 5500 and 19500 units of IFN, respectively.

Fig. 2. Time course of inhibition of IFN yield by 2-AP. Primary chick embryo cells aged in vitro for 8 days were induced to make IFN by infecting them with u.v.-irradiated avian reovirus at an m.o.i. of 5. 2-AP was added to the medium immediately after infection (time = 0 h), kept in for the time interval shown on the abscissa, and then washed out. Fresh medium was added and incubation at 40.5 °C continued for a total of 24 h. The medium was then assayed for its IFN content. Control yields of IFN from a monolayer of $1 \times 10^7$ cells in a 50 mm dish were 18500 units.

Fig. 3. Time at which 2-AP acts to block IFN induction. Monolayers of aged chick embryo cells were infected with u.v.-irradiated avian reovirus at an m.o.i. of 5 and 2-AP (10 mM) was added to the medium at the times indicated by arrows, and kept in for the duration of the experiment. After a total of 24 h the medium was harvested and assayed for its content of IFN. A plot of the fraction of control yield of IFN against the time of addition of 2-AP produced the curve represented by the solid circles (●). A similar curve was generated in which AcD (0.1 μg/ml) was added in place of 2-AP (○). The normal time course of IFN induction by u.v.-inactivated avian reovirus on aged chick embryo cells is shown for comparison (◇). Control yields of IFN were 15000 units per $10^7$ cells.

the IFN-inducing particle, and incubated for 4 h prior to wash-out of the drug, there was a 95% reduction in IFN yield. Fifty percent reduction of yield was achieved after 1 h exposure to 2-AP. Similar results were observed with mouse L(Y) cells as the host and VSV-tsG31 as the IFN-inducing particle (data not shown).

2-Aminopurine acts early during IFN induction

Fig. 3 shows that 2-AP acts early during IFN induction. The curve fitted to the solid circles was generated by measuring the yield of IFN at 24 h as a function of the time at which 2-AP was added to the cell cultures after the start of infection (induction). When aged chick embryo cells were infected with u.v.-inactivated avian reovirus and 10 mM-2-AP was added immediately thereafter there was a 95% reduction in IFN yield (time 0). If addition of the drug was postponed
Table 1. Interferon yields from chick embryo cells treated transiently with 2-AP or AcD prior to interferon induction

<table>
<thead>
<tr>
<th>Drug</th>
<th>Time in drug (h) prior to removal and IFN induction*</th>
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</thead>
<tbody>
<tr>
<td>None (mock)</td>
<td>7200† 4800 9600</td>
</tr>
<tr>
<td>2-AP (10 mM)</td>
<td>7200 4800 7200</td>
</tr>
<tr>
<td>AcD (50 ng/ml)</td>
<td>2700 720 180</td>
</tr>
</tbody>
</table>

* The IFN-inducing particle was u.v.-inactivated avian reovirus at an m.o.i. of 5. IFN was collected after 20 h post-infection and incubation at 40.5 °C.
† Represents the IFN yield in total units from $1 \times 10^7$ aged chick embryo cells.

until 6 h post-infection, maximum (control) yields of IFN were obtained 24 h later, even in the continued presence of the drug. Addition of the drug at intervals prior to 6 h resulted in correspondingly lower yields of IFN, with a 50% reduction in yield if the drug was withheld for the first 3 h. This curve in Fig. 3 represents the escape of IFN induction from the action of 2-AP (solid circles), and appears similar to that reported for the action of actinomycin D (AcD) on IFN induction (reviewed in Stewart, 1981). When AcD actually was substituted for 2-AP in the experiment described, an essentially identical escape curve was generated (Fig. 3, open circles). As a control for the time course of IFN induction in these experiments, a third curve was generated (Fig. 3, crossed circles). The last two results confirmed those reported by Svitlik (1985).

The inhibitory action of 2-aminopurine on IFN induction is reversible

The similarity of the IFN yield-escape curves for the action of 2-AP and AcD, and the irreversible nature of the latter's action, prompted a test for the reversibility of the action of 2-AP on IFN induction. Monolayers of aged chick embryo cells were exposed to 2-AP or AcD for different periods of time prior to wash-out of the drug and induction of IFN with u.v.-inactivated avian reovirus. Table 1 illustrates that chick cells exposed for 2 to 6 h to a concentration of 2-AP (10 mM) that reduced the yield of IFN by 95% if present for 4 h immediately after virus infection, produced control yields of IFN when induction was carried out after wash-out of the drug. Thus, the action of 2-AP appears to be completely reversible. In contrast, the action of AcD appears to progress with time and is long lasting. [It should be noted that a relatively low concentration of AcD (50 ng/ml) was used in these experiments so that the cells' integrity would not be affected during the 24 h incubation period for IFN production].

Aged chick cells tolerate continuous exposure to 2-aminopurine and become phenotypically altered hosts for plaque formation

Experience with 2-AP suggested that concentrations of the drug that have profound effects on IFN inducibility might not be deleterious to the cell. Consequently, we determined whether 2-AP present in an agar overlay would alter the phenotype of aged chick cells by rendering them more sensitive to plaque formation by viruses. Fig. 4(a) shows that monolayers of aged chick cells do not normally support plaque formation by VSV-New Jersey (Hazelhurst), a good inducer of IFN which is also sensitive to its action (Marcus et al., 1987). However, when 2-AP (10 mM) was included in the agar overlay this virus formed plaques with high efficiency (Fig. 4b). The background of uninfected cells stained vitally with neutral red shows that prolonged exposure to 2-AP had no adverse effect on cell viability.

Effect of 2-aminopurine on two other inducible genes

Two other inducible gene systems were tested for their response to 2-AP to determine the breadth of the drug's action.
2-Aminopurine blocks interferon induction

Fig. 4. Plaque formation by VSV-New Jersey (Hazelhurst) in chick embryo cells treated, or not, with 2-AP. Monolayers of primary chick embryo cells were aged in vitro for 7 days. Control cell monolayers were inoculated with a 10⁻⁶ dilution of a wild-type stock of VSV-New Jersey (Hazelhurst) and covered with a standard agarose overlay (a). Treated cell monolayers were inoculated with the same dilution of virus, but covered with an agarose overlay that contained 10 mM-2-AP (b). All plates were incubated at 37.5 °C for 3 days, vitally stained with neutral red, and then photographed with Kodachrome 64 to produce a 'positive' negative. Consequently, plaques appear as dark areas against a light background. Illustrations are presented at actual size. The central area in the plate of control cells (a) represents a discontinuity in the monolayer, not plaques.

Fig. 5. Virus yield reduction curves generated by treating cells with IFN in the presence of 2-AP. Mouse L cells (○, ●) and chick embryo cells (■, □) were treated with different amounts of their respective IFNs for 24 h at 37.5 °C in the presence of 10 mM (●, □) or no (○, ■) 2-AP. Following IFN treatment, the monolayers were challenged with wild-type VSV-Indiana (HR) and the yield of VSV p.f.u. was determined 12 h later. Control yields of VSV from chick embryo and mouse L cells were 2.7 x 10⁹ and 6.4 x 10⁹ p.f.u./ml, respectively. The yields of virus from chick embryo and mouse L cells that were exposed to 2-AP, but not to IFN, for the time equivalent of the IFN treatment, were 1.4 x 10⁹ and 2.8 x 10⁹ p.f.u./ml, respectively. Actual concentrations of mouse and chicken IFN represented by 1.0 on the abscissa were 46 and 7 units per ml, respectively.

Interferon action

Monolayers of aged chick embryo cells, or mouse L cells, were treated with different concentrations of their respective species of IFN for 19 h in the presence of 2-AP. The IFN and drug were then removed, and the cells challenged with wild-type VSV. Twenty-four h later the
Table 2. Activation of an interferon-mediated antiviral state is not inhibited by the action of 2-AP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Virus yield (p.f.u./ml)</th>
<th>Fraction of control</th>
</tr>
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<tbody>
<tr>
<td>None (control)</td>
<td>$1.4 \times 10^9$</td>
<td>1.0</td>
</tr>
<tr>
<td>IFN*</td>
<td>$5.6 \times 10^6$</td>
<td>0.0038</td>
</tr>
<tr>
<td>2-AP†</td>
<td>$2.7 \times 10^9$</td>
<td>1.9</td>
</tr>
<tr>
<td>IFN + 2-AP</td>
<td>$7.4 \times 10^6$</td>
<td>0.0028</td>
</tr>
</tbody>
</table>

* Monolayers of chick embryo cells were treated with 10 units/ml chick IFN for 24 h at 37.5 °C, washed, and challenged with VSV-Indiana (HR). Virus was harvested at 12 h post-infection and assayed for p.f.u. activity.
† 2-AP at 10 mM was added to cells after the IFN treatment, and during the virus challenge.

It was still possible that 2-AP might affect activation of the antiviral state following virus challenge, especially if it involved PKIFN dsRNA (Rice et al., 1985). Consequently, an experiment was performed in which 2-AP was added after IFN treatment, and was present during the entire period of virus challenge. The results are shown in Table 2; they document an approx. 300-fold reduction in VSV yield in both IFN- and IFN- plus 2-AP-treated cells, thus demonstrating that activation of the antiviral state upon virus challenge was not affected by this drug.

Heat shock (stress) proteins

Chick embryo cells exposed to heat shock or other stress conditions are induced to make a series of stress proteins, the most prominent being SP88 and SP71,72 (Hightower, 1980). When 2-AP (10 mM) was included in the medium bathing chick embryo cells at the time of heat shock (44 °C, 2 h), there was no discernible effect on the induction of stress proteins as observed by one-dimensional PAGE (data not shown).

DISCUSSION

The phosphorylation–dephosphorylation of proteins can function to control their activity, and thus regulate intracellular processes (Krebs & Beavo, 1979). In the interferon system there is one protein kinase of potential interest to our understanding of the regulation of IFN induction, a dsRNA-activated kinase that phosphorylates eIF2-α and a 69K ribosome-associated protein, P1 (Samuel, 1987). This protein kinase (PKIFN dsRNA), with its high affinity binding to dsRNA (Hovanessian & Kerr, 1979), was proposed as a putative host factor involved in recognizing dsRNA at an early step in IFN induction on the path to derepression of the IFN gene(s) (Marcus, 1984). PKIFN dsRNA is inhibited strongly by 2-AP (Farrell et al., 1977), and this actually reverses the loss of translational activity in a cell-free system caused by the phosphorylation-mediated inactivation of eIF2-α. The inhibition of PKIFN dsRNA by 2-AP allows endogenous phosphoprotein phosphatases to convert eIF2-α from the phosphorylated inactive form to the dephosphorylated active form (DeBenedetti & Baglioni, 1983). If 2-AP acts in vivo as it does in vitro, and phosphorylation regulates IFN induction, then the ratio of protein kinase to phosphoprotein phosphatase may regulate IFN induction, including variation in the ratio of IFN types produced.

Data presented here demonstrate that 2-AP has a selective effect in blocking IFN induction, in both chick embryo and mouse L(Y) cells. A virus containing preformed genomic dsRNA failed to induce IFN, indicating that 2-AP does not act by blocking formation of dsRNA. Two other cellular systems involving inducible genes, namely those for IFN action and for stress (heat shock) response, were unaffected by the action of this drug.

Concentrations of 2-AP in the range of 1 to 2 mM often stimulated IFN induction about twofold, suggesting that induction may be finely regulated through the degree of protein phosphorylation. Yet higher concentrations of 2-AP consistently prevented IFN induction in a
2-Aminopurine blocks interferon induction

dose-dependent manner (Fig. 1). Concentrations of 2-AP in excess of 15 mM maintained on cells for 24 h, or longer, often had a deleterious effect on their integrity. However, cells need only be exposed to 2-AP for 4 h after infection for the maximum effect of the drug on IFN induction to be expressed (Fig. 2). This period encompasses the time required to deliver an inducer molecule of dsRNA to the cell's interior (Svitlik & Marcus, 1985) and initiate transcription of the IFN genes (Stewart, 1981). This implicates a stage in IFN induction presumed to encompass interaction of the proximal inducer molecule (dsRNA) and the putative cellular receptor (IFN induction receptor), that precedes transcription of the IFN gene(s). Further evidence for 2-AP acting early during IFN induction, and at a pretranscriptional level, comes from data showing that IFN induction escapes the action of 2-AP at the same rate as it escapes the action of AcD, the latter representing a well-defined inhibitor of DNA-dependent RNA synthesis (Fig. 3). These results also indicate that once transcription is completed, translation of the IFN mRNA is not affected by 2-AP. Indeed, 2-AP can actually enhance translation by activating phosphorylated eIF2-α through dephosphorylation (DeBenedetti & Baglioni, 1983). Conceivably this reaction underlies the enhanced production of IFN at low concentrations of 2-AP (Fig. 1).

One remarkable feature of the action of 2-AP in blocking IFN induction is its total reversibility. Unlike the action of AcD [or indomethacin (Sekellick & Marcus, 1985), cycloheximide (Svitlik & Marcus, 1985) and IFN induction-suppressing particles (Marcus & Sekellick, 1987a) in chick embryo cells], once 2-AP is removed from the cell, the cell rapidly resumes its normal response to viral inducers of IFN (Table 1). Clearly, 2-AP must be present only during the critical early time when the IFN inducer molecule of dsRNA interacts with cellular components, for example the postulated IFN induction receptor (Marcus, 1984).

As reported previously (Carver & Marcus, 1967; Sekellick & Marcus, 1985), primary chick embryo cells aged in vitro for several days are capable of producing such high levels of IFN that viruses which share the attributes of being both good inducers of IFN and sensitive to its action fail to develop plaques on these host cells. This characteristic feature of aged chick embryo cells can be abolished by including 2-AP (10 mM) in the agar overlay: plaque formation then proceeds normally (Fig. 4). This dramatic change in host cell phenotype demonstrates that concentrations of 2-AP that inhibit IFN induction significantly do so selectively, leaving other vital attributes of the cell unaffected, i.e. its capacity to replicate virus and stain with neutral red (Fig. 4).

Analysis of the effect of 2-AP on both facets of IFN action revealed that it did not affect either the development of the antiviral state (Fig. 5), or its activation by viruses, presumably by dsRNA (Table 2), known to activate dsRNA-dependent enzymes of the IFN system in vivo (Rice et al., 1985).

How might 2-AP act to block IFN induction? If 2-AP functions as an inhibitor of protein kinase in these experiments, as it does in vitro (Farrell et al., 1977), then we may speculate on a role for the kinase in IFN induction. As proposed earlier (Marcus, 1983, 1984), the high affinity binding of PK_{IFN-dsRNA} to dsRNA makes it a likely molecule to serve as an interferon induction receptor, the first cellular component thought to interact with a molecule of dsRNA as it enters, or is formed, in the cytoplasm. Since one molecule of dsRNA may activate several molecules of PK_{IFN-dsRNA}, and the latter may undergo several catalytic rounds of activation (Schneider & Shenk, 1987), dsRNA interaction with the protein kinase may generate an amplified signal. The activated protein kinase may serve to phosphorylate other molecules thereby further amplifying the initial interaction, an event apparently required to ensure the high efficiency of IFN induction achieved with one molecule of dsRNA per cell (Marcus & Sekellick, 1977). The simplest model may ultimately involve phosphorylation of the factors bound to the regulatory region of the IFN gene (Zinn & Maniatis, 1986). Phosphorylation of these factors may alter their configuration or otherwise act to reduce their binding to regulatory sites on the DNA, thereby releasing repressor molecules and allowing function of the trans-acting factor required for activation of the IFN gene (Enoch et al., 1986). Indeed, several proteins are activated (or inactivated) upon phosphorylation. For example, in yeast cells, a heat shock factor that binds constitutively to DNA may activate transcription only after heat-induced phosphorylation (Sorger et al., 1987).
Double-stranded RNA can induce IFN and it can activate PK_{IFN-dsRNA}. The structural features of dsRNA required for these two reactions are similar. The IFN-inducible PK_{IFN-dsRNA} displays high affinity binding for dsRNA, and under certain conditions both IFN induction and PK_{IFN-dsRNA} activation share an unusual dose-response to dsRNA. These observations, coupled with data presented here, support a model in which PK_{IFN-dsRNA} may function as a putative IFN inducer receptor by interacting with dsRNA as the proximal IFN inducer molecule and, through modulation of phosphorylation, constitute a critical early step in IFN induction.

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REFERENCES


2-Aminopurine blocks interferon induction


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