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## Perspective

# Modulation of Leukocyte Genetic Expression by Novel Purine Nucleoside Analogues. A New Approach to Antitumor and Antiviral Agents

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Stimulation of the immune system has long been recognized to be highly effective in the prevention and the treatment of viral infection.[1] Also, recent studies clearly indicate the feasibility and the usefulness of cancer treatment by immunostimulation, either by the use of vaccine[2] or cytokine induction[3] or by adoptive immunotherapy with lymphokine-activated killer cells or tumor-infiltrating lymphocytes.[4] On the other hand, lymphatic leukemia therapy may involve specific induction of leukocyte death.[5] Modulation of the immune system response appears to be a great challenge for the next years, and it now appears that purine nucleoside analogues can play interesting roles in such modulation.

The syntheses of nucleoside analogues have filled and will continue to fill the research and development pipeline of many pharmaceutical firms for several decades. If progress in anticancer[6,7] and antiviral[8] chemotherapy can be attributed in large part to this extensive work in medicinal chemistry, challenges to produce new nucleoside analogues with more specific site and mechanism of action remain. Recently, some adenosine and guanosine analogues have been synthesized and determined to be potent and specific modulators of leukocyte promotion/proliferation/differentiation/death processes. Such modulation of the immune system, inhibition by adenosine analogues and stimulation by guanosine analogues, is discussed in this paper.

### Adenosine Analogues as Antileukemic Agents

1-β-Arabinofuranosylcytosine (*ara*-C, Cytarabine) is widely used in clinic for its antileukemic activity, and many other β-D-arabinofuranosyl nucleosides have been synthesized and tested for their antiviral and/or antineoplastic properties.[9,10] 2-Chloro-2′-deoxyadenosine (2-CdA, 1) and 9-β-D-arabinofuranosyl-2-fluoroadenine (2-F-*ara*-A, fludarabine, 2) (Chart I) have been shown to be highly specific in their action against proliferative diseases which affect cells of the immune system and are now used in clinic with high response rates and low toxic effects.

### 2-Halogeno-2′-deoxyadenosines

2-CdA was first synthesized in 1972,[11] and a direct synthesis from 2,6-dichloropurine which gives only the β-anomer has been reported from our laboratory in 1984.[12] 2-CdA has also been prepared enzymatically from 2-chloroadenine and thymidine, as the deoxyribose donor, by using a nucleoside transdeoxyribosylase purified from extracts of *Lactobacillus helveticus*[13] or *Lactobacillus leichmanii*.[14]

2-CdA has been shown to be highly active, at nanomolar concentrations, in the inhibition of various human malignant T or B lymphocytes[11,13,15-17] in vitro. The reported $ID_{50}$ values against the T lymphoblastoid cell line CCRF-CEM and the B lymphoblastoid cell line WI-L2 were 3 and 35 nM, respectively.[13] Likewise, 2-CdA has exhibited $ID_{50}$ values of 70 and 42 nM[18] against the L1210 and P388 leukemia cells, respectively. Furthermore 2-CdA, at a concentration of $10^{-7}$ M, has been reported to inhibit the proliferative response of peripheral blood mononuclear cells to polyclonal activators as well as to allogeneic cells in a mixed lymphocyte culture.[19] In 1983,[15] experiments on nondividing human peripheral blood lymphocytes, for the first time, pointed out the unique property of 2-CdA to induce lethality in resting cells. Monocytes, which are considered as nondividing cells, have also been shown to be very sensitive to 2-CdA, the $IC_{50}$ being as low as 27 nM after 5 days of culture.[20] As for lymphocytes, toxicity is

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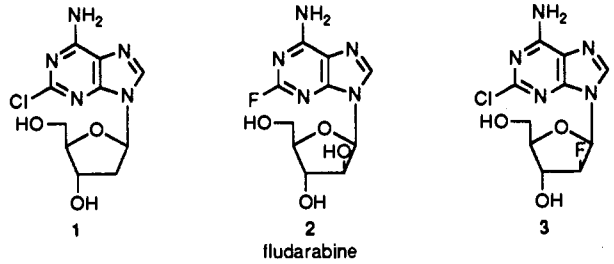
**Chart I.** Adenosine Derivatives with Immunoinhibitor Potencies




both dose and time dependent. It is now clear the cytotoxicity of 2-CdA, similar to 2′-deoxyadenosine, is independent of cell division.[21] So far, evidence that 2-CdA is highly active against dividing as well as some resting leukocytes suggests this nucleoside does not behave as a classical antimetabolite. It is of interest that 2-CdA has demonstrated no in vitro cytotoxicity against nondividing, confluent fibroblasts.[20] 2-CdA has also been shown to be much less inhibitory to cell lines isolated from human solid tumors than against T or B lymphoblasts.[22] The concentrations required to exhibit inhibitory activity toward breast, colorectal, kidney, lung, or stomach tumor specimens were at least 100-fold higher than the nanomolar concentrations determined to inhibit by 50% T or B lymphoblastoid cells survival.[22] Recently, a marked and dose-dependent toxicity of 2-CdA to human myeloid progenitor cells has also been reported.[23] The $\beta$-anomer of 2-CdA has been shown to be essential for its cytotoxicity as the concentrations of the $\alpha$- and $\beta$-anomers required to provide 50% growth inhibition of leukemia L1210 cells were as different as $10^{-4}$ M for the $\alpha$ and $7 \times 10^{-8}$ M for the $\beta$, respectively.[11] Early structure–activity relationship studies have demonstrated the much lower inhibition of the 2-methoxy, 2-methylthio-,[11] and 2-fluoro-2′-deoxyadenosine (2-FdA).[24] Like 2-CdA, 2-bromo-2′-deoxyadenosine (2-BrdA) has been shown to be a potent inhibitor of the proliferation of T lymphoblastoid, B lymphoblastoid, and myeloid cell lines in culture.[16] 2-BrdA has exhibited very high in vitro cytotoxicity to human lymphoblastic CCRF-CEM cells with an $EC_{50}$ value of 12 nM, that is however 4 times the value determined for 2-CdA, although the $EC_{50}$ value determined for 2-FdA was much higher at 150 nM.[24]

The in vitro inhibitory activity of 2-CdA has been confirmed by in vivo studies. For instance, 2-CdA was shown to protect mice from L1210 leukemia mortality.[13] Intraperitoneal injections of 50 mg/kg 2-CdA once a day for 6 days following tumor inoculation afforded an 81% increase in life span (ILS) in mice.[13] This value was slightly improved (91% with 99.98% of cell kill) when 2-CdA was administered every 3 h × 8 one day after tumor implantation.[16] When given more frequently every 3 h for 8 days at 10 mg/kg per dose 2-CdA gave 5/5 60-day survivors.[18] These in vivo studies have clearly pointed out the importance of continued dosing and prolonged treatment with 2-CdA. The high efficacy of multiple-dose regimens could be explained by a rapid metabolism and a short half-life of this nucleoside in mice, in accord with data available from recent pharmacokinetic studies in humans.[25] When given ip to L1210-inoculated mice at 40 mg/kg per dose every 3 h × 8 on days 1, 5, and 9, 2-BrdA and 2-FdA afforded 50% and 30% survival rates, respectively, at day 40.[24] In this in vivo test, 2-CdA was shown to be superior to 2-BrdA and 2-FdA. When treated according to the same schedule with only 15 mg/kg per dose of 2-CdA, 60% of the leukemic mice were still alive at day 40.[24] In fact similar increases in life span following a 3 h × 8 one-day treatment have been reported with 2-BrdA and 2-CdA, but optimal dosage was lower for 2-CdA.[16] An extensive study with 2-BrdA has confirmed that the optimal activity against L1210 leukemia in mice required multiple doses given every 3 h (×8) according to an appropriate schedule on days 1, 4, and 7.[26] Such a regimen afforded an ILS value of 417%. 2-BrdA has also been tested in drug combinations against the same murine leukemia. The highest ILS values were obtained in association with aziridinylbenzoquinone, 5-azacytidine, or 1-$\beta$-D-arabinofuranosylcytosine (353%, 225%, and 223%, respectively).[17] 2-BrdA alone has been shown to inhibit the M5076 ovarian carcinoma and the B16 melanoma in mice (ILS of 46% and 66%, respectively).[26]

## Clinical Use of 2-Chloro-2′-deoxyadenosine in Lymphoid Malignancies

The first clinical trial with 2-CdA was reported by Carson et al. in 1984.[27] The study showed this nucleoside was well tolerated and was effective in patients with low-grade malignant disorders of the lymphoid tissue, including chronic lymphocytic leukemia or non-Hodgkin's lymphoma. Higher doses than 0.1 mg/kg per day were shown by this phase I trial to induce bone marrow suppression and to increase susceptibility to infection. Since 1988,[28] many studies have confirmed the unique efficiency of this nucleoside at the clinical level on different lymphoid malignancies with low growth rate: advanced chronic lymphocytic leukemia of B-cell origin,[28] hairy cell leukemia (HCL),[28,29] cutaneous T-cell lymphomas,[30] or low-grade non-Hodgkin's lymphomas.[31] As suggested by the studies on animals, a several-day treatment has appeared necessary to optimize the action of the drug. Phase II trials with 2-CdA given by slow infusion at a classical dose of 0.1 mg/kg per day for 7 days have highlighted the lack of potent toxicity usually associated with standard treatment by other aggressive cytotoxic antitumor chemotherapeutic agents. The in vitro potent antimonocyte activity of 2-CdA[20] has been however confirmed, since cytopenia, with a constant decrease to 0 of the monocyte count,[32] has appeared to be a common side effect encountered during 2-CdA treatment. These clinical trials have also shown patients with lymphopenia or decreased T-cell number might be considered as patients at risk of infection.[32] Nevertheless, due to the unique efficacy and relative safety of 2-CdA during phase II clinical trials, Piro,[33] in a recent editorial in *Blood*, emphasizes the great interest and clinical importance of the treatment by this drug in lymphoproliferative disorders. Up to date clinical data[32-34] on the treatment of patients with HCL by 2-CdA, raise the overall response rates to values as high as 81–95% and the complete remission rates to values between 75% and 85%. Recovery from cytopenia is faster (2–10 weeks, depending on the cell line) than what is usually expected from interferon therapy (2–5 months).[32] If a single course of continuous intravenous therapy for 7 days at the dose of 0.1 mg/kg per day was initially considered as the treatment of choice, recent pharmacokinetic[25] and clinical data[32] demonstrate the possibility of intermittent infusion. Oral or subcutaneous treatment by 2-CdA can also be expected in future studies.[32] Clinical results with different purine analogues, 2-CdA, fludarabine, and 2′-deoxycoformycin, have been analyzed and emphasized by Cheson[35] in a recent

editorial in the *Journal of Clinical Oncology* entitled "The Purine Analogues—A Therapeutic Beauty Contest". These three purine analogues are reported by the author to "have proven to be some of the most interesting compounds to be developed in recent years". The National Cancer Institute has recently made 2-CdA available in the United States for patients with active HCL through a group C treatment protocol (NCI 191-0019).

2-CdA has also been evaluated in phase I and II trials in children and young adults with relapsed acute myeloid leukemia and acute lymphoid leukemia.[36,37] Treatment by continuous infusion for 5 days showed 2-CdA was significantly active against acute myeloid leukemia with a total response rate of 59%. Furthermore, 47% of the patients underwent complete hematologic remissions. However 2-CdA-induced toxicity was notable, with systematic severe neutropenia and thrombocytopenia. Further clinical evaluation in multidrug regimens against acute myeloid leukemia is warranted.

## Mechanism of Action of 2-CdA

The exact mechanism of action of 2-CdA which is responsible for the clinical antitumor effect is unknown. Similarities have been shown between the cytotoxicity induced by the 2-halogeno-2′-deoxynucleosides which are resistant to deamination by adenosine deaminase and the immunodeficiency disease observed in the inherited deficiency of this enzyme.[15,21] Adenosine deaminase catalyzes the hydrolytic deamination of adenosine and deoxyadenosine.[38] Lymphopenia linked with adenosine deaminase deficiency may be due to the toxic accumulation of deoxynucleotides within the cell. Toxicity of 2′-deoxynucleosides toward malignant human T-cell lines has been attributed to high levels of deoxynucleoside phosphorylating activity, as determined on the cell line CCRF-CEM, and low levels of deoxynucleotide dephosphorylating activity, as determined on the three cell lines CCRF-CEM, CCRF-HSB, and MOLT-4.[39] In a similar manner, in sensitive cells, 2-CdA is phosphorylated to the monophosphate form by deoxycytidine kinase and accumulates as the nucleoside triphosphate.[13] Observation of this phosphorylation stage has been demonstrated in malignant lymphoblasts[13] as well as in nondividing monocytes[20] or human peripheral blood lymphocytes.[15,21] Deficiency in deoxycytidine kinase[13] or supplementation with deoxycytidine[13,20,21,40] protected the cells from toxicity.

2-CdA inhibits DNA synthesis in vitro and in vivo.[13,16] The $IC_{50}$ values measured on diploid CCRF-CEM cells were 24 nM after 4-h exposure and 16 nM after 18-h exposure.[16] Two mechanisms have been shown to be directly involved: (i) the imbalance of the deoxynucleoside triphosphate pools attributed to the inhibition of ribonucleotide reductase by 2-CdATP which results in less competition of dATP with 2-CdATP during DNA synthesis and more incorporation of 2-CdAMP into tumor DNA,[41] and (ii) the inhibition of DNA polymerases by 2-CdATP which limits DNA extension.[42,43] These two mechanisms lead to a self-potentiation of the effects.

Studies on mouse mammary tumor FM3A cells have shown dATP and dGTP intracellular pools were largely depleted as soon as 3 h after treatment with 20 μM 2-CdA.[41] However all deoxynucleotide pools were not equally affected. In fact an imbalance was observed and dTTP pools increased as dCTP pools remained unchanged. This imbalance has been considered by several authors to be a key factor in the toxicity of 2-CdA.[41,42] An endonuclease, which induces double-strand breaks in DNA, has been shown to be activated by 2-CdA.[40] Hirota et al.[41] have suggested the imbalance of the deoxynucleotide triphosphate pools was the cause of such an activation of the endonuclease. Unfortunately no data concerning this hypothesis has since been reported. The depletion of dATP pools has been correlated with the potent inhibition of the ribonucleotide reductase activity by 2-CdA at nanomolar concentrations after its conversion to the triphosphate form.[42] This enzymatic inhibition leads to a marked decline in ADP reduction and consequently a decrease in the intracellular levels of dATP. This decrease is thought to be important in aiding the competition of 2-CdATP with dATP for incorporation into the A sites of growing DNA strands.[42]

Parker et al.[42] found in 1988 that 2-CdA was incorporated into the 3′ end of the native DNA strands and elongation beyond these substitution points was retarded due to an inhibition of either polymerase α or β by 2-CdATP. The former result was confirmed by Hentosh et al.[43] in 1990, in that 2-CdA was incorporated in growing DNA chains into many positions coding for adenosine. However Hentosh et al. reported different sensitivities for the inhibition of DNA polymerases α and β.[43] In contradiction with Parker's results, 2-CdA was found to be a very weak inhibitor of human polymerase α. A significant decrease in chain extension was confirmed using DNA polymerase β which may be the enzyme primarily responsible for DNA repair synthesis.[43] After 30 min of incubation with human polymerase β, the progress in the extension of a synthetic primer was decreased from a maximum of more than 240 bases added to the initial nucleotide sequence in the presence of 10 μM dATP to a maximum of only 60 bases added in the presence of the same dosage of 2-CdATP. In comparison, the maximum number of bases added using human polymerase α was only decreased from approximately 120 with 10 μM dATP to 80 with 10 μM 2-CdATP. Studies with the other 2-halogeno-2′-deoxyadenosines have shown 2-BrdA poorly substituted for dATP using either human polymerase α and β, although 2-FdA was able to completely substitute for the natural substrate with polymerase α and γ but not with polymerase β.[42] Using polymerase α or β, 2-BrdA was found to decrease the DNA chain extension rate at sites of multiple consecutive insertion more than with 2-CdA.[43] Interestingly, the 2-halogeno-2′-deoxynucleotides have exhibited inhibition of DNA primase activity only at very high concentrations ($IC_{50}$ ranging from 80 μM for BrdATP to 220 μM for FdATP).[42]

A partial decrease in RNA synthesis has been reported to be induced by 2-CdA in lymphocytes[16,21] as well as in mouse mammary tumor FM3A cells[41] and monocytes.[20] This inhibition has been attributed to a probable result of the primary DNA fragmentation. To date no study concerning a possible direct effect of 2-CdA on RNA polymerases, as is shown with fludarabine,[44] has yet been described.

A marked depletion in NAD concentration has also been shown in 2-CdA-treated cells compared to normal.[20,21] This decrease, attributed to the consumption of NAD for the formation of poly(ADP-ribose) which activates the DNA repair synthesis by DNA ligase, has been reported to be the principal cause of death in normal resting lymphocytes.[21] However further studies in monocytes have

demonstrated 3-aminobenzamide, an ADP-ribosylation inhibitor, prevented the NAD depletion but did not block the process of cell death.[20] Since the NAD cellular concentration remained constant during the first 4 h, depletion in the NAD pools was only seen as a consequence of the DNA damage.[20] Recent studies in lymphocytes have confirmed that supplementation of 2-CdA-treated cells with nicotinamide could not prevent the initial DNA breakdown.[40]

In lymphocytes[21] as well as in monocytes,[20] 2-CdA has been reported to disturb the natural equilibrium between spontaneous natural breakage and the current repair of these lesions in DNA. 2-CdA inhibits DNA repair in resting lymphocytes.[45] In mouse mammary tumor cells, 2-CdA has been established to affect DNA double strands without significant effect on DNA single strands.[41] In X-irradiated Chinese hamster V79 cells, recent studies have confirmed 2-CdA inhibited the repair of double-stranded DNA.[46] It seems unlikely topoisomerases I and II are involved in this inhibition since 2-CdA, as opposed to 2-BrdA, has been shown to be virtually ineffective in the inhibition of these enzymes.[47] In the same model of X-irradiated Chinese hamster cells, 2-CdA did not affect DNA chain elongation.[46] These results suggest that inhibition of the repair of DNA strand breaks by 2-CdA can not be directly correlated with the termination of DNA chain biosynthesis.[46]

## A Program of Cell Death

When exposed to micromolar concentrations of 2-CdA, lymphocytes[40,48] as well as monocytes[40] or thymocytes[49] have been shown to die from apoptosis. This type of cell death is a major mode of death for eukaryotic cells,[50-53] and the name apopotosis (from the Greek "falling off") was first proposed by Kerr et al.[54] Apoptosis is a "programmed cell death" which naturally occurs in endocrine tissue atrophy, normal tissue turnover, or in some stages of embryogenesis and plays an important role in development and growth regulation.[53] Apoptosis is observed in the process of killing cells by cytotoxic T lymphocytes, where, after a first stage of recognition, the target cell is inevitably programmed for death. Apoptosis is also likely to occur in T or B cells in order to regulate the immune response.[53] Although this process of cell death, conversely to necrosis, is usually not the result of an injury, it can be initiated by different stimuli such as, for example, in the glucocorticoid-induced apoptosis of thymocytes[55] or chronic B lymphocytic leukemia cells.[56] Apoptosis appears as the result of a developmental program, and many features are characteristic of the pathway to death of apoptotic cells. In the early stages, adhesion of the cell with its neighbors is lost and the nucleus appears more condensed with formation of peripheral dense crescent-shaped aggregates of chromatin along the nuclear membrane. Cell contraction is due to cytoplasmic protein condensation and loss of cytoplasmic volume. These morphological changes are concomitant with chromatin fragmentation. There is internucleosomal cleavage of chromatin with formation of typical DNA double-strand fragments whose lengths are multiples of 180–200 base pairs. This DNA breakdown provides a useful biochemical marker of the apoptotic process as it leads to a distinctive ladder pattern on electrophoretic gels. After these morphological and biochemical events, the cell breaks down into many membrane-bound apoptotic bodies which frequently contain nuclear components. Finally these fragments are either sloughed off or phagocytized and completely degraded by adjacent cells and macrophages. This type of cell death concerns single cells and not groups of cells as in the case of necrosis. It does not induce an inflammatory response or any damage to neighboring cells. Unlike necrosis, apoptosis always appears as an active process which requires energy to maintain cellular integrity as well as RNA and protein synthesis to allow progression through the different stages leading to death. 2-CdA-treated leukocytes present many hallmarks of apoptosis.[40,48,57] Very early, within the first hours, treatment with 1 $\mu$M 2-CdA leads to an initial accumulation of DNA strand breaks.[40] After 12–18 h, DNA gel electrophoresis shows the ladder pattern distinctive of internucleosomal fragmentation. Loss of viability and cell lysis begin to be observed 24 h after supplementation of the culture with 2-CdA.[40]

The exact mechanism by which the early accumulation of DNA strand breaks originates in 2-CdA-treated lymphocytes, thymocytes, and monocytes, as well as mammary tumor cells or even log-phase Chinese hamster V79 cells remains to be defined. Many studies have shown primary DNA damage induced by 2-CdA, further followed by the activation of a $Ca^{2+}$–$Mg^{2+}$ endonuclease,[40] can be prevented by cycloheximide, an inhibitor of protein biosynthesis.[41,46,48] In mouse mammary tumor cells, this inhibition of DNA breakdown was followed by restoration of cell viability.[41] Likewise, cycloheximide has been shown to inhibit DNA cleavage and consequent cell death in glucocorticoid-[55] or deoxyadenosine-[59] treated thymocytes. However, cycloheximide did not prevent cell death in 2-CdA-treated Chinese hamster V79 cells, although it inhibited accumulation of double-strand breaks.[58] This lack of protection is probably due to the higher dose of 2-CdA (5 $\mu$M) necessary to induce signs of toxicity in this cell line.[58] At this dose, 2-CdA may interfere with biochemical pathways other than those normally involved in more sensitive cells. Inhibition of DNA damage afforded by cotreatment with cycloheximide indicates that an early expression and/or transcription of one or more protein which deregulates the normal equilibrium within the cell and programs it for death is implicated in the mechanism of action of 2-CdA.

Recent studies have shown many factors can be involved in this apoptosis. Enhancement of Jun/AP-1 activity and subsequent induction of c-*jun* transcription is observed upon treatment of myeloid leukemia cells with arabinofuranosylcytosine, which has also been determined to induce apoptosis.[60] The roles of the activator protein-1 (AP-1) and of the protooncogene c-*jun* are yet to be clarified in the regulation of promotion/proliferation/differentiation of these cells[61,62] and their implication as intermediate agents in the apoptotic process is still controversial. TGF$\beta_1$, the transforming growth factor, has been shown to promote this form of cell death in human hepatoma[63] or gastric carcinoma[64] cell lines. The inhibitory effect of cycloheximide on the cytocidal potency of TGF$\beta_1$ treatment was reported to be either nonsignificant[63] or dose dependent.[64] Thus, it appears possible that, in leukocytes, 2-CdA could promote apoptosis via induction of TGF$\beta_1$ gene expression.

Another protein, *Bcl*-2, has also been implicated in the glucocorticoid-induced apoptosis of some pre-B lymphocytes.[65] Internucleosomal DNA damage was observed after glucocorticoid treatment only when low levels of *Bcl*-2

were expressed in cells. This result confirmed the fact the product of the *Bcl*-2 oncogene was able to block apoptosis, as it has been shown in other cell lines.[66-70] Thus, *Bcl*-2 underexpression seems to be necessary for the apoptosis process to develop.

Protein p53, which has been identified as an growth/tumor suppressor gene product,[71,72] is naturally expressed in the nonproliferative, mature B cells, T cells, granulocytes, and monocytes where it could play a role by inhibition of cell proliferation.[73] There is no evidence that the p53 protein is a mediator of apoptosis in normal cells but it has been recently reported that (1) levels of p53 protein highly increased after DNA damage induced by different exogenous agents ($\gamma$-irradiation, actinomycin D,[74] UV, or UV mimetics[75]) and may play a role in the inhibition of DNA synthesis that follows DNA breakdown,[74] (2) induced expression of transfected wild-type p53 gene-promoted apoptosis in a human colon tumor-derived cell line,[76] (3) transfection of the wild-type p53 gene in murine myeloid leukemic cells which do not naturally express p53 protein induced apoptosis.[77] This process was inhibited by interleukin 6 (IL-6).[77] In this latter model, IL-6 acts as an inactivator of a tumor suppressor gene that could mediate apoptosis. Early studies have shown low concentrations of 2-CdA reduced the release of IL-6 in normal monocytes[20] and it is also known that glucocorticoids can repress the IL-6 promotor region of the IL-6 gene.[78,79] Furthermore, phorbol esters have been shown to totally inhibit DNA fragmentation in 2-CdA-treated HCL cells[57] as well as in 2-CdA-[48] or glucocorticoid-[56] treated chronic lymphocytic leukemia cells. Phorbol esters can be seen as inhibitors of protein kinase C but it has also been found that the human IL-6 gene contained two second messenger-responsive enhancer (MRE I and MRE II) regions which can be independently strongly stimulated by phorbol esters to activate IL-6 transcription.[80] Finally, IL-6 has recently been found to prevent glucocorticoid-induced apoptosis in a human myeloma cell line.[81] It is to date still speculative to assess that specific inhibition of IL-6 expression is related to the induction of apoptosis by 2-CdA but this hypothesis must surely be further considered. In any case, it appears that the primary mechanism of action of 2-CdA may involve direct or indirect regulation of the expression of specific genes whose products play a role in the induction of such programmed cell death.

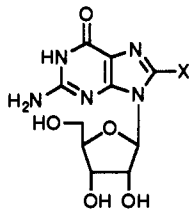
## Fludarabine

9-$\beta$-D-Arabinofuranosyl-2-fluoroadenine 5′-monophosphate (F-*ara*-AMP, fludarabine phosphate) was introduced into the clinic in 1983 and has recently been approved in the United States by the Food and Drug Administration. Thus fludarabine phosphate is presently readily available for the treatment of patients with relapsed or refractory chronic lymphocytic leukemia. A concise review from H. G. Chun et al. of the National Cancer Institute on the properties of this anticancer agent has recently appeared.[82]

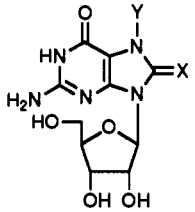
Fludarabine (2), first synthesized in 1969 by Montgomery et al.,[83] is formulated as the 5′-phosphate which is rapidly dephosphorylated to fludarabine before crossing cellular membranes.[82] F-*ara*-AMP enters the cell as the nucleoside form and rapidly undergoes intracellular rephosphorylation via deoxycytidine kinase.[84,85] Like 2-CdA, fludarabine induces apoptosis in lymphocytes[48] and thymocytes.[49] DNA damage is followed by inhibition of DNA synthesis. Fludarabine is also a potent inhibitor of ribonucleotide reductase activity.[84,86] 2-F-*ara*-ATP competes with dATP for incorporation into growing DNA strands and is a potent inhibitor of polymerases $\alpha$.[86,87] Thus, extensive insertion of 2-F-*ara*-AMP at the 3′-termini of native DNA results in termination of chain elongation.[88] Fludarabine inhibits RNA metabolism.[44] The decrease of the RNA biosynthesis activity appears to be mostly due to the inhibition of RNA polymerase II. Fludarabine is incorporated into the growing RNA chain, and its insertion induces premature termination of the RNA transcript and disrupts protein synthesis.[44] Selectivity of action against leukemia cells seems to be due to a high incorporation rate in proliferative cells via a facilitated single carrier-mediated diffusion process followed by higher levels of phosphorylation in tumor cells than in normal cells.[89] Interestingly, fludarabine and 2-CdA have been recently shown to prevent recombinant interleukin 2-induced proliferative responses of peripheral blood mononuclear cells to polyclonal or to allogeneic activators.[90] These two nucleosides have also been shown in mice to stimulate natural killer cell activity at doses (75 and 25 mg/kg per day for 3 days, respectively) that clearly reduce primary antibody response to T-dependent or T-independent antigens.[91,92] Stimulation by fludarabine may occur via an adenosine P intracellular receptor.[93] As for 2-CdA, the exact mechanism of induction of apoptosis by fludarabine is yet unknown.

Recently, new analogues of fludarabine have been synthesized and their antiproliferative properties evaluated.[94,95] In vitro determination of the $ID_{50}$ of 2-chloro-9-(2-deoxy-2-fluoro-$\beta$-D-arabinofuranosyl)adenine (Cl-F-*ara*-A, 3) (Chart I) against human peripheral blood lymphocytes (28 nM) and monocytes (47 nM) has proved the high potency of this compound against these leukocytes, although values determined for 2-CdA were nearly 2-fold lower.[96] Cl-F-*ara*-A appears to be a potent inhibitor of a variety of human tumor cell lines (ACHN renal carcinoma, SNB-7 CNS tumor, NCI-H23 non-small-cell lung adenocarcinoma, SK-MEL-28 melanoma) at submicromolar concentrations as well as of K562 chronic myelogenous leukemia cells ($IC_{50}$ = 28 nM).[97] Cl-F-*ara*-A provided promising results in vivo, with good increases in life span (+220%) in mice inoculated with P388 leukemia.[94] Cl-F-*ara*-A was also shown to be curative against both early and advanced stage (14 days) murine colon 36 solid tumor with 5/6 tumor free survivors.[97] Unlike 2-CdA, Cl-F-*ara*-A was stable in acid at pH 2.[96] This finding was in agreement with a study by Marquez et al.[98] who demonstrated introduction of a fluorine atom at the 2′ position of the arabino ring highly inhibited hydrolysis of the glycosidic bond.[98] Furthermore, Cl-F-*ara*-A resisted enzymatic degradation by *Escherichia coli* nucleoside phosphorylase. These unique properties led Carson et al.[96] to compare the bioavailability and activity of Cl-F-*ara*-A and 2-CdA when given to mice orally at a concentration of 1 mg/mL during 1 week. By this route, murine plasma concentrations were 10-fold higher with Cl-F-*ara*-A (562 nM) than with 2-CdA (48 nM) and, similarly, measures of the inhibitory activity against chronic lymphocytic leukemia were in favor of Cl-F-*ara*-A. Phosphorylation by deoxycytidine kinase of Cl-F-*ara*-A to Cl-F-*ara*-AMP, which is further metabolized into the triphosphate form, was shown to be required for activity. Cl-F-*ara*-ATP was demonstrated to be a potent inhibitor of ribonucleotide

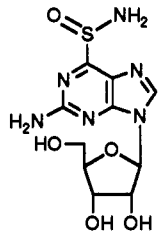
**Chart II.** Guanosine Derivatives with Immunostimulant Potencies



**4:** X = Br
**5:** X = SH
**6:** X = OH



**7:** X = O, Y = $CH_3$
**8:** X = O, Y = $CH{=}CHCH_3$ (loxoribine)
**9:** X = S, Y = $CH_3$
**10:** X = Se, Y = $CH_3$
**11:** X = O, Y = $CH_2CH_2CH_3$



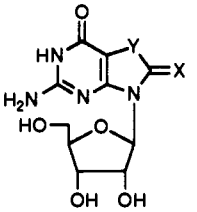
**30**
sulfinosine

reductase activity and depressed deoxyribonucleoside triphosphate pools, except TPP pools.[95,99] The incorporation of Cl-F-*ara*-AMP into a newly synthesized DNA strand resulted in termination of DNA chain elongation using both DNA polymerase $\alpha$[95,99] and $\epsilon$.[99] Cl-F-*ara*-A did not inhibit DNA polymerase $\beta$, $\gamma$, or DNA primase.[95] It induced marked decrease in DNA synthesis with an $IC_{50}$ of 50 nM as measured in human K562 cells.[95] DNA strand breaks were determined in monocytes as well as in normal or leukemic lymphocytes between 4 and 8 h after the onset of the culture with Cl-F-*ara*-A. Like 2-CdA and fludarabine, Cl-F-*ara*-A was shown to induce apoptosis.[96] In view of its similarities with 2-CdA and fludarabine and due to its unique oral bioavailability, this newly described adenosine analogue could have a very promising future.

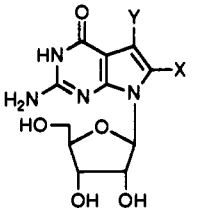
## In Vitro Studies of the Immunostimulant Potency of Some Guanosine Type Nucleosides

**Leukocyte Proliferation Induction.** Many guanosine derivatives and analogues positively modulate the proliferation and/or differentiation of leukocytes. 8-bromoguanosine[100] (8-BrGuo, **4**), 8-mercaptoguanosine[100] (8-SHGuo, **5**), and 8-hydroxyguanosine[101] (8-OHGuo, **6**) (Chart II) have been first synthesized in our laboratory. 8-BrGuo has been shown to stimulate CBA/CaJ spleen murine cells mitogenesis in vitro.[102] 8-SHGuo clearly enhanced B-cell proliferation selectively with no mitogenic activity on T lymphocytes.[103] Maximum proliferation of mature mouse splenic B cells occurred with 1 mM 8-SHGuo. All responsive cells were characterized by the presence of the surface phenotype $\mu^+$ $FcR^+$ Thy-1.2 and most of them possessed surface Ia antigen, $\delta$-chain, C3 receptors, and Lyb3/5/7 antigens.[103] The presence of T cells did not enhance the proliferative response. In contrast to mouse B cells, rat B cells were shown to be unresponsive to the mitogenic potency of 8-SHGuo.[104] Likewise, 8-BrGuo and 8-SHGuo were unable to induce human B cells or peripheral blood mononuclear cells to proliferate.[105,106] 8-BrGuo and 8-hydroxyguanosine were less potent than 8-SHGuo in the stimulation of murine B-cell proliferation.[107] However, 8-BrGuo and 8-SHGuo

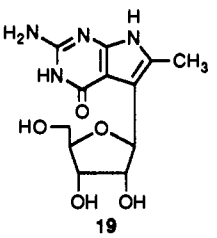
**Chart III.** Guanosine Analogues



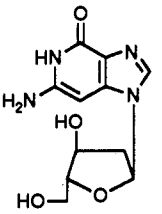
**12:** X = O, Y = S



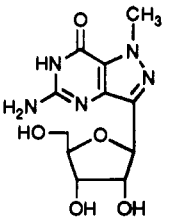
**13:** X = H, Y = H
**14:** X = H, Y = COOH (cadeguomycin)
**15:** X = H, Y = CN
**16:** X = H, Y = $CONH_2$
**17:** X = H, Y = phenyl
**18:** X = Cl, Y = H



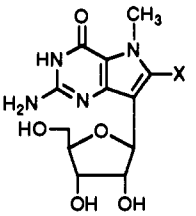
**19**



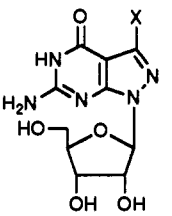
**20**



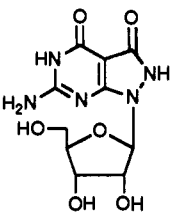
**21**



**22:** X = Cl
**23:** X = Br



**24:** X = H
**25:** X = Br
**26:** X = $NH_2$
**27:** X = $CH_3$
**28:** X = $OCH_3$



**29**

have been shown to be much less potent than 7-methyl-8-oxoguanosine (7-Me-8-oxoGuo, **7**) (Chart II) and 5-amino-3-$\beta$-D-ribofuranosylthiazolo[4,5-*d*]pyrimidine-2,7-(3*H*,6*H*)-dione[108,109] (7-thia-8-oxoguanosine, 7-thia-8-oxoGuo, **12**) (Chart III). These latter two guanosines were first synthesized in our laboratory and reported in 1969[110] and 1990,[109] respectively. The preparation of 7-Me-8-oxoGuo was improved in 1987,[111] in order to obtain sufficient quantities for further in vivo experiments. The more recently synthesized guanosine analogue, 7-thia-8-oxoGuo, significantly stimulates the proliferation of murine CBA/CaJ spleen cells at concentrations as low as 10 $\mu$M after 48-h incubation. In 1991, the direct synthesis of 7-allyl-8-oxoguanosine (loxoribine, **8**) by substitution of 8-BrGuo with the sodium salt of allyl alcohol followed by thermal polyhetero-Claisen rearrangement was reported.[112] Loxoribine has been found to greatly enhance the proliferative response of murine CBA/CaJ spleen cells, with a maximal stimulation occurring at a concentration of 0.3 mM.[113] In agreement with the absence of mitogenic activity of 8-BrGuo and 8-SHGuo on human B cells, loxoribine was reported to be ineffective in inducing a proliferative response in cultures of human peripheral blood mononuclear cells.[114] Interestingly, 8-methoxyguanosine, 8-aminoguanosine,[107] and 7-deazaguanosine (7-deazaGuo,[115] **13**) did not exhibit any B- or T-cell proliferative potency.

Guanosine derivatives or analogues failed to cause direct proliferation of T lymphocytes in the absence of other mitogenic signal. 7-Thia-8-oxoGuo was ineffective in activating T cells[116] and 7-deazaguanosine did not exert any proliferative effect on human peripheral blood lym-

phocytes.[117] 8-SHGuo has been reported by Goodman and Weigle to promote murine thymocyte proliferation in the presence of either IL-2, alloantigenic irradiated spleen cells,[118] or soluble protein recall antigen.[105] Conversely, Feldbush and Ballas[104] have shown this nucleoside was unable to enhance the proliferation of murine thymocytes even when cultures were exposed to various costimulators such as phytohemagglutinin A, IL-1, or recombinant IL-2. 8-SHGuo was even shown by these authors to be a slight inhibitor of the IL-1 response.

A series of 3-deazaguanine nucleosides was synthesized in 1984[119] and evaluated in 1990 as immunomodulating agents.[117] The $\alpha$-nucleoside $\alpha$-2′-deoxy-3-deazaguanosine ($\alpha$-d3DGuo, **20**) displayed the most potent immunoenhancing potency on activated T-cell response.[117] Phytohemagglutinin or concanavalin A-induced T-cell proliferation was markedly enhanced in a dose-dependent manner by $\alpha$-d3DGuo concentrations ranging from 0.0125 to 0.4 mM. The $CD4^+$ and not the suppressor $CD8^+$ T lymphocyte subpopulation appeared to be specifically stimulated by $\alpha$-d3DGuo. This nucleoside was unable to stimulate resting T cells to proliferate but was a potent stimulator of the clonal expansion of activated T lymphocytes. It was furthermore able to restore the cyclosporin A-depressed proliferative response of T lymphocytes in vitro. No adjuvant effect of $\alpha$-d3DGuo was seen on B-cell proliferation, even in response to a specific mitogen. The $\beta$-anomer exhibited both immunopotentiating and immunosuppressive effects on T-lymphocyte proliferation, depending upon the dose and the level of activation of the lymphocytes.[117]

**Activation of Leukocyte Differentiation.** Activation of B-cell differentiation by guanosine derivatives is highly documented and concerns different cell lineages of various mammalian species.[120] 8-BrGuo was shown by Goodman and Weigle[121] in 1982 to induce polyclonal activation of immunoglobulin secretion in murine CBA/CaJ spleen cells. The in vitro activation of these cells, as measured by the generation of plaque forming cells in response to sheep red blood cells (SRBC) or to 2,4,6-trinitrophenol, was maximum at a concentration of 1 mM.[121] 7-Me-8-oxoGuo is 10 times superior to 8-BrGuo or 8-SHGuo in its ability to induce primary humoral immune response in mouse spleen cells,[122] and its potent adjuvant properties have been confirmed in human lymphoid cells.[123] In human B cells, 8-BrGuo and 8-SHGuo have been shown to induce a 10 times larger immunoglobulin production than pokeweed mitogen although a 10 times smaller production than Epstein–Barr virus.[106] IgG, IgA, and IgM secretion were equally enhanced and the maximal polyclonal B-cell response occurred in the presence of T helper cells. These in vitro studies on human cells confirmed that a maximal immunoglobulin secretion required the presence of T helper function. However it is clear this requirement is not necessary to induce a primary activation of the B cells, and this activation can be T cell-independent.[124,125] Furthermore, enhancement by 8-SHGuo of polyclonal IgG and IgA as well as antigen-specific antibody secretion have been reported in human Epstein-Barr virus-infected peripheral blood lymphocytes depleted of T cells.[126] In purified rat B cells also, despite the absence of T cells and regardless of the fact that 8-SHGuo did not induce any proliferation, a marked increase in IgM and a modest increase in IgG were observed.[104] This ability of 8-SHGuo to induce B-cell differentiation without proliferation guided Feldbush and Ballas[104] to suggest this C8-substituted guanosine exhibited B cell differentiating factors $\mu$- and $\gamma$-like activities. The finding that 8-SHGuo as well as 8-methoxyguanosine[127] can stimulate B-lymphocyte differentiation without concomitant effect on proliferation demonstrates the specificity on B-cell populations by the various guanosine nucleosides. Likewise, 8-SHGuo has been shown by Feldbush and Ballas to have no clonal expansion effect on murine T cells but to be able to promote differentiation of these cells in response to allogeneic antigens, by inducing the generation of specific cytotoxic T lymphocytes.[104]

Many in vitro studies have pointed out certain unique properties of these guanosine derivatives which are able to induce immunopotentiation even when added late to the culture medium. CBA/CaJ splenic B cells have been supplemented with 8-SHGuo up to 48 h after the onset of the culture without effect on the adjuvanticity of this guanosine derivative.[124,128] 7-Me-8-oxoGuo could also be added on the third day of a 6-day culture of human peripheral blood lymphocytes without modification of the immunopotentiation.[108,123]

The adjuvant properties of 8-SHGuo have been evaluated in cultures of hyporesponsive leukocytes from senescent and neonatal mice. This guanosine was able to restore a nonspecific humoral immune response in spleen cell cultures from senescent mice as measured by an anti-SRBC plaque-forming cells assay.[108] However failure of complete restoration of normal immunity was attributed to a disruption in the interactions between senescent B and T cells.[108] Spleen cells from neonatal mice were shown to be activated by 0.3 mM 8-SHGuo, and the antigen-specific response was comparable with that of mature B cells in the absence of adjuvant potentiation.[125] Likewise, in cultures of human neonatal B cells, the level of IgM secretion was enhanced by 3-fold in the presence of 0.5 mM 8-SHGuo.[129] Interestingly, the effect of the guanosine derivative was maximum when added 3 days after onset of the culture. All these in vitro studies confirm the efficacy of these guanosine derivatives as important adjuvants of the immune response.

These nucleosides have also been shown in vitro models to exhibit a potential for overcoming different kinds of immunodeficiencies. 8-SHGuo was able to restore the immune responsiveness of B cells from immunodeficient mice with the x-linked immune defect.[127] These B cells, which are normally nonresponsive to the TNP-Ficoll antigen, were shown to respond to this T cell-independent type 2 antigen in the presence of 0.25 mM 8-SHGuo and even in the absence of serum components in the culture medium. Moreover, if 8-SHGuo was added 24 h after the antigen, the immune response was still highly effective.[127] 7-Me-8-oxoGuo has also been recently tested for its ability to generate an immune response from lymphocytes of patients with common variable immunodeficiency. With doses of 7-Me-8-oxoGuo ranging from 0.3 to 1 mM, the in vitro response was in most of the cases comparable or higher to that elicited by normal cells.[130]

Although 8-SHGuo was shown to influence the response of immature B cells, it did not seem to be able to stimulate resting B cells in phase $G_0$.[127] Studies on murine spleen cells have demonstrated that all B cells were not equally affected by 8-SHGuo or 8-BrGuo. The primary target of these compounds has appeared to be large activated B cells which could directly undergo proliferation and

differentiation.[131] Resting B cells were not induced to proliferate or differentiate upon addition of guanosine derivatives alone but were shown to be rendered sensitive to the adjuvant activity of these nucleosides after stimulation by other signals. Arguing with the nature of these signals, Ahmad and Mond[127] have pointed out the essential role of mitogens or specific immunogens. Goodman[132] has emphasized the costimulatory role of B lymphotropic cytokines present in mixed lymphocyte culture or concanavalin A-induced supernatants. Although in early studies[108,123] a preparation of IL-2 was shown to synergize with 7-Me-8-oxoGuo or 8-SHGuo to induce immune response, later experiments[132] with purified fractions have demonstrated neither IL-1, IL-2, IL-3, IL-4, IL-5, granulocyte-macrophage colony stimulating factor nor interferon (IFN)-$\gamma$ was able to potentialize the adjuvant activity of 8-SHGuo. Conversely IFN-$\alpha$ and IFN-$\beta$ were shown to exhibit antigen-dependent synergistic properties. Nevertheless, as the synergy between 8-SHGuo and mixed lymphocyte culture or concanavalin A-induced supernatants was not annihilated by anti-IFN-$\alpha/\beta$ antibody, it was concluded that these two interferon subtypes were not the only factors which could further enhance the adjuvanticity of 8-SHGuo.[132]

It is clear from these in vitro studies guanosine derivatives enhance the initial immune response of large activated B cells. This primary adjuvanticity is T cell-independent and guanosine derivatives have been shown to replace, to some extent, the requirement for cytokines.[104,124] Nevertheless, this first immune activation is further increased by the recruitment of new B-cell populations due to the participation of yet still undetermined T-cell activating signals. As far as some cytokines are concerned, other leukocytes like macrophages or natural killer (NK) cells might not be excluded from this adjuvant process.[116,133-135]

The adjuvant properties of guanosine derivatives are not limited to B or T lymphocytes. In vitro studies have shown NK cells and macrophages were also activated when murine spleen cells were supplemented with 8-BrGuo at doses as low as 75 or 37.5 $\mu$g/mL respectively.[133] This activation was shown to be dependent on an induced IFN production in the culture medium. A direct action of 8-BrGuo and 7-thia-8-oxoGuo on macrophages has been demonstrated in vitro, since these guanosine derivatives were able to activate phagocytosis in vitro and to stimulate generation of superoxide anion, as measured by a chemiluminescence assay, even in the absence of other stimulators such as zymosan particles or phorbol esters.[135] 8-BrGuo was also able to induce an IL-1 like activity in cultures of the macrophage cell line P388D$_1$[134] and 8-SHGuo synergized with very low doses of IL-2 to induce the in vitro generation of lymphokine-activated killer (LAK) cells from NK-like precursors.[136] These LAK cells were cytotoxic toward NK-sensitive and NK-resistant tumor cells but not normal lymphoblasts in vitro. Likewise, loxoribine has been shown to reduce the concentration of IL-2, IFN-$\alpha$, or IFN-$\gamma$ required for the induction of human LAK cell cytotoxicity.[114] Recently, the T cell-specific activator $\alpha$-d3DGuo was shown to induce the generation of cytotoxic lymphocytes in a dose-dependent manner in allogeneic mixed cultures of peripheral blood mononuclear cells.[117]

## In Vivo Studies of the Guanosine Derivatives and Analogues

In vivo animal studies have confirmed the ability of many known and novel guanosine derivatives to enhance the overall immune response. Various guanosines have now been demonstrated to have a major in vivo effect in animal models of viral infections and in the treatment of metastases.

The in vivo adjuvant effect of 8-BrGuo was recognized by Goodman and Weigle[137] as early as 1983 in CBA/CaJ mice.[137] A significant increase in specific plaque-forming cells was demonstrable at a high dose of 10 mg/mouse when 8-BrGuo was injected with the antigen. The optimal dose was lower (0.3 mg/mouse) if its administration was delayed 3 days after immunization.[137] Likewise, 8-SHGuo was shown by Mond et al.[138] in 1989 to be active in mice by enhancing $IgG_1$, $IgG_2$, and $IgG_3$ levels in response to TNP Ficoll with no effect on IgM production.

Guanosine derivatives may not only enhance immunity but are also able to extend or restore the immune response. The two earliest studied C8-substituted guanosines, 8-BrGuo and 8-SHGuo, for example, were able to initiate the antibody response of immune-defective *xid* mice,[137,138] and antibody-dependent cellular cytotoxicity was shown to be restored by 7-thia-8-oxoGuo in homozygous beige mutant mice.[139] Guanosines can also interrupt nonresponsive states of the immune system. In certain physiological cases, T cells can become tolerant to an antigen, called tolerogen, and the immune response is then abrogated. B cells can no longer receive signals from T cells and do not produce any antibody against this tolerogen. 8-BrGuo has been shown to be able to terminate this tolerant state 60 days after its induction, when T cells but not B cells remain tolerant.[140] This in vivo activation of the B-cell response bypasses the ineffectiveness of T cells and suggests 8-BrGuo can deliver a T-helper-like signal to B lymphocytes. In a model of aged CBA/CaJ mice, 7-Me-8-oxoGuo was shown to enhance the in vivo antibody response to aggregated human $\gamma$ globulin[141] and restore a subpopulation of senescent B cells to become sensitive to the tolerization process.[142] 7-Thia-8-oxoGuo was also able to enhance antibody-dependent cellular cytotoxicity in aged mice.[139] These in vivo results have confirmed that certain guanosine derivatives can act in a positive manner to improve various immunodeficiencies, especially in the case of aging and the natural decline of the immune system.

In vitro and in vivo experiments have both demonstrated a clear activation of the humoral immune response by guanosine derivatives. However, recent studies[109,135,139,143-145] have mainly been concerned with the potency of these compounds to enhance cellular immunity, by the generation of killer cells from lymphocytes and/or the activation of phagocytosis in monocytes/macrophages. This unique property has been well documented in vivo since the synthesis reported in 1990 by our laboratory of a series of thiazolo[4,5-*d*]pyrimidine nucleosides.[109] One of them, 7-thia-8-oxoGuo, was shown to not only enhance the humoral immune response in vitro, by promoting B-cell proliferation at nearly micromolar concentrations, but also to dramatically activate NK cell and macrophage activity in mice.[109,143,144] Certain guanosine analogues, including 7-thia-8-oxoGuo, can activate zymosan particle- or phorbol ester-induced phagocytosis in vivo,[135] and the ability of 7-thia-8-oxoGuo to enhance antibody-dependent cellular cytotoxicity of both mac-

rophages and lymphocytes against P815 tumor cells in mice has been clearly demonstrated.[139] Likewise, loxoribine has been reported to enhance NK cell- and macrophage-mediated cytotoxicity toward B16 melanoma cells in mice.[145]

## Antiviral and Antitumor Inhibition by Various Substituted Guanosine Derivatives and Analogues

All the in vitro and in vivo data accumulated during the last decade on guanosine derivatives have proved their unique ability to enhance T-cell, B-cell, macrophage, or NK-cell differentiation. The enhancement of the immune response is the key mechanism of action of certain of these guanosines which have been shown to exhibit excellent antiviral and/or antitumor activity in vivo.

**Antiviral Potency.** Although 8-BrGuo and 8-SHGuo are very weak or modest antiviral agents,[109] many other positive immunomodulators such as 7-thia-8-oxoGuo (**12**) or 7-deazaguanosine (**13**) have demonstrated considerable antiviral potency in vivo. The most striking fact is that such active antiviral agents of this guanosine type do not exhibit any direct antiviral activity in virus-infected cells in vitro but act dramatically by enhancing the overall immune response of the host.

7-Me-8-oxoGuo exhibited greater in vivo antiviral activity against Semliki Forest virus (SFV) infection than did 8-BrGuo or 8-SHGuo. In a mouse model, 8-BrGuo was inactive against this viral infection; 8-SHGuo at 100 mg/kg afforded 50% (6/12) survivors at 21 days compared to no survivors in the control.[109] In the same experiment 200 mg/kg 7-Me-8-oxoGuo gave 75% survivors.[109] The recently synthesized 7,8-dihydro-7-methyl-8-thioxoguanosine[146] (7-Me-8-thioGuo, **9**) and 7,8-dihydro-7-methyl-8-selenoxoguanosine (7-Me-8-selenoGuo, **10**, Chart II) exhibited comparable antiviral activities against SFV infection in mice.[147] When administered at 50 mg/kg ip 24 and 18 h before virus infection, 7-Me-8-oxoGuo gave 67% (8/12) survivors compared to no survivors in the control untreated mice. 7-Me-8-thioGuo and 7-Me-8-selenoGuo provided 83% (10/12) and 58% (7/12) survivors, respectively. 5-Amino-1-methyl-3-$\beta$-D-ribofuranosylpyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one (**21**), synthesized in 1991,[148] is a C-nucleoside analogue of these 7-methylguanosines and has been shown to induce lymphocyte proliferation at 1 $\mu$M concentration in human peripheral blood mononuclear cell cultures. Its efficacy against SFV infection in mice was of the same level of potency as reported for 7-Me-8-oxoGuo.[148]

7-Me-8-oxoGuo and 7-Me-8-thioGuo also offered very good protection against banzi and San Angelo viruses infections in mice with an average of 90% survivors when given in half-daily intraperitoneal doses of 25 or 50 mg/kg.[146] Furthermore, these compounds protected mice against encephalomyocarditis (EMC) virus mortality (75% survivors at 50 mg/kg for both of those derivatives).

7-Thia-8-oxoGuo has appeared in in vitro and in vivo studies to be a very potent immunostimulant with regard to its ability to activate B-cell proliferation and differentiation as well as macrophages and NK cells cytotoxicity. 7-Thia-8-oxoGuo exhibits high in vivo activity against a number of RNA viral infections. It is much more potent than 8-BrGuo, 8-SHGuo, and 7-Me-8-oxoGuo in the protection of mice against SFV infection with 92% (11/12) survivors at 21 days when given in divided daily doses of 100 mg/kg or 200 mg/kg ip.[109] 7-Thia-8-oxoGuo is also highly protective in mice or rats against San Angelo, banzi, Punta Toro, cytomegalovirus, rat coronavirus, and EMC virus.[149-152] Treatment of a San Angelo virus infection in mice could be deferred 24 h after the virus challenge with a significant antiviral effect (75% survivors vs 100% when given 2 h before).[149] In a murine model of Friend leukemia virus-induced splenomegaly, total protection could be obtained with divided doses of 50 mg/kg 7-thia-8-oxoGuo at day 2, 6, 9, 13, and 16 after virus inoculation.[150] Unfortunately, delayed regimens were ineffective against SFV infection.[150] 7-Thia-8-oxoGuo has exhibited weak to moderate protection against herpes simplex viruses type 1 and 2 and vesicular stomatis virus.[149,150]

**Table I.** Activity of Guanosines Active against Rat Coronavirus Infection in Suckling Rats

| compd | dose[a] (mg/kg) | survivors total (%) | mean survival time[b] (days) |
|---|---|---|---|
| placebo[c] | | 1/11 (9) | 7.4 ± 2.5[d] |
| 7 | 100 | 9/12 (75)[e] | 9.7 ± 0.6 |
| 9 | 100 | 7/12 (58)[e] | 9.4 ± 2.3 |
| 12 | 100 | 10/12 (83)[e] | 8.0 ± 0.0 |
| 18 | 100 | 11/12 (92)[e] | 7.0 ± 0.0 |

[a] Half-daily intraperitoneal doses were administered 24 and 18 h before virus inoculation. [b] Mean survival time of mice that died; survivors lived through 21 days. [c] A 2% sodium bicarbonate solution served as the placebo and as diluent for the compounds. [d] Standard deviation. [e] Statistically significant ($p < 0.05$), determined by the two-tailed Fischer exact test.

The suckling rat coronavirus model is a small animal model of human coronavirus infection and is predictive of antiviral activity against the human common cold symptoms due to this infection. In this experimental model, 7-thia-8-oxoGuo,[149,150] 7-Me-8-oxoGuo, and 7-Me-8-thioGuo,[146] given at a dose of 100 mg/kg 24 and 18 h before virus challenge were shown to be highly protective (Table I) against rat coronavirus infection. The best result with 7-thia-8-oxoGuo (95% survivors) was obtained with two divided doses of 100 mg/kg ip given 4 and 8 h after viral infection in the same rat coronavirus model.[150] The intranasal route has also been explored for treatment of rhinovirus and coronavirus infections.[151] When administrated intranasally to rats 24 and 18 h before virus inoculation, 0.3% and 1% concentrations of 7-thia-8-oxoGuo were highly protective against coronavirus lethality in suckling rats.[151] These findings suggest that these guanine derivatives deserve further study in the human treatment of the common cold.

Since 7-thia-8-oxoGuo was effective against San Angelo virus infection when given prior to or 24 h after virus inoculation, further tests were performed against another virus of the family of bunyaviruses. Punta Toro virus is a member of this negative-stranded RNA virus family and is more specifically a representative of the group of phleboviruses to which belong important lethal pathogens such as Rift Valley fever virus and sandfly fever virus. Punta Toro virus is a closely related less virulent virus which is a good model for the evaluation of antiphlebovirus agents. 7-Thia-8-oxoGuo exhibited high prophylatic and therapeutic activities against the hepatotropic Adames strain of Punta Toro virus.[152] Divided doses were more protective than full daily doses as they maintained a higher degree of immunoenhancement for a longer period of time. Initiation of the treatment in order to insure overall reduction of the disease parameters and complete protection at 21 days could be deferred as late as 36 h after virus infection.[152] Further explorations of the efficacy of

Table II. Activity of 7-Deazaguanosines against Semliki Forest Virus Infection in Mice

| compd | dose (mg/kg) | treatment route[a] | survivors total (%) | mean survival time[b] (days) |
|---|---|---|---|---|
| 13 | 0[c] | ip | 1/12 (8) | 7.4 ± 2.2[d] |
| | 50 | ip | 10/12 (83)[c] | 6.0 ± 0.0 |
| | 100 | ip | 10/12 (83)[c] | 6.5 ± 0.7 |
| | 200 | oral | 10/12 (83)[c] | 8.0 ± 0.0 |
| 14 | 0 | ip | 2/12 (17) | 6.8 ± 2.1 |
| | 100 | ip | 1/12 (8) | 8.1 ± 2.4 |
| | 200 | ip | 4/12 (33) | 6.0 ± 1.4 |
| 15 | 0 | ip | 1/12 (8) | 6.0 ± 0.8 |
| | 100 | ip | 6/12 (50) | 8.5 ± 3.0 |
| 18 | 0 | ip | 1/12 (8) | 6.5 ± 1.3 |
| | 25 | ip | 11/12 (92)[c] | 6.0 ± 0.0 |
| | 50 | ip | 12/12 (100)[c] | >21 |
| | 100 | ip | 11/12 (92)[c] | 7.0 ± 0.0 |

[a] Half-daily doses were administered 24 and 18 h before virus inoculation. [b] Mean survival time of mice that died; survivors lived through 21 days. [c] A 2% sodium bicarbonate solution served as the placebo and as diluent for the compounds. [d] Standard deviation. [e] Statistically significant ($p < 0.05$), determined by the two-tailed Fischer exact test.

Table III. Activity of 7-Deazaguanosines against San Angelo Virus Infection in Mice

| compd | dose[a] (mg/kg) | survivors total (%) | mean survival time[b] (days) |
|---|---|---|---|
| 13 | 0[c] | 4/12 (33) | 7.9 ± 1.8[d] |
| | 25 | 11/12 (92)[e] | 9.0 ± 0.0 |
| | 50 | 12/12 (100)[e] | >21 |
| | 100 | 12/12 (100)[e] | >21 |
| 18 | 0 | 2/11 (18) | 7.2 ± 1.6 |
| | 10 | 7/12 (58) | 8.0 ± 1.0 |
| | 25 | 12/12 (100)[e] | >21 |
| | 50 | 12/12 (100)[e] | >21 |

[a] Half-daily intraperitoneal doses were administered 24 and 18 h before virus inoculation. [b] Mean survival time of mice that died; survivors lived through 21 days. [c] A 2% sodium bicarbonate solution served as the placebo and as diluent for the compounds. [d] Standard deviation. [e] Statistically significant ($p < 0.05$), determined by the two-tailed Fischer exact test.

7-thia-8-oxoGuo and related guanosine derivatives in the treatment of serious bunyavirus infections are warranted.

7-Deazaguanosine (7-deazaGuo, **13**), an analogue of the antibiotic cadeguomycin **14** (Chart III), was first synthesized in 1976 by Townsend, Robins, and co-workers.[153] An improvement of the preparation by a stereospecific sodium salt glycosylation procedure has been recently reported by Robins, Revankar, and co-workers.[154] 7-DeazaGuo has no effect in vitro on T- or B-cell proliferation.[117] In vivo studies in mice have demonstrated the very high potency of this compound enhances phagocytosis and activates NK cell cytotoxicity.[115] In the presence of zymosan as a costimulator of phagocytosis, doses of 50 mg/kg ip of 7-deazaGuo were 2 times more potent than equivalent doses of 8-BrGuo or 7-thia-8-oxoGuo in activating phagocytic cells. This activity ratio was increased to 5-fold in the absence of zymosan.[115] NK-cell activation by 7-deazaGuo in mouse spleen cells following divided daily oral and ip treatments was 2- or 3-fold over that of untreated controls.[115] 7-DeazaGuo is the first orally active guanosine analog which shows significant inhibition against RNA viruses in vivo. 7-DeazaGuo was more potent than 7-thia-8-oxoGuo in its ability to induce protection against SFV and San Angelo virus infection in mice. The highest activities (83% and 100% survivors with SFV and San Angelo virus, respectively) were obtained with doses as low as 50 mg/kg per day ip. Similar protection levels (83% and 92%, respectively) were conferred to mice by oral treatment with higher doses.[115] Oral protection by 7-deazaGuo was also demonstrated against banzi and EMC viral infections.[115] The 7-cyano and 7-carboxylic acid (cadeguomycin) derivatives, **15** and **14**, respectively, of 7-deazaGuo (**13**) are less active than the parent compound against SFV infection in mice (Chart III and Table II) although 7-carboxamido and 7-phenyl derivatives, **16** and **17**, respectively, are totally inactive (data not shown). Likewise, replacement of the ribofuranosyl moiety by arabinofuranose or 2′-deoxyribofuranose in cadeguomycin **14** leads to inactive compounds in this antiviral test (data not shown). However, another derivative, 2-amino-6-chloro-7-β-D-ribofuranosyl-2*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**18**), when given ip, exhibited higher protection than 7-deazaGuo against SFV (Table II) as well as San Angelo virus (Table III) infection in mice. Interestingly, **18** was also shown to be slightly more potent than 7-thia-8-oxoGuo against rat coronavirus infection in suckling rats (Table I). A positional isomer of 7-deazaguanosines, 2-amino-6-methyl-5-β-D-ribofuranosyl-2*H*-pyrrolo[2,3-*d*]-pyrimidin-4(3*H*)-one (**19**), was totally inactive against SFV infection.[155]

Some 9-deazaguanosine derivatives (Chart III) have recently been synthesized by direct stereospecific β-C-glycosylation of 2-aminopyrrolo[3,2-*d*]pyrimidin-4(3*H*)-one (9-deazaguanine).[155] Antiviral efficacies of these guanosine analogues were evaluated against SFV infection in mice. Although the parent 9-deazaguanosine and its 7-methyl derivative did not show any activity, 8-chloro-7-methyl-9-deazaguanosine (**22**) given ip at the dose of 100 mg/kg protected 83% of the animals, compared to 92% with 7-thia-8-oxoGuo at the same dosage. 8-Bromo-7-methyl-9-deazaguanosine (**23**) was less active with 58% survivors with the same standard protocol.[155]

6-Amino-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (**24**) and its 3-substituted derivatives **25–29** (Chart III) can be considered as guanosine analogues with the nitrogen atom in position 7 of the guanosine moved to position 8. Synthesis, in vivo effect on murine NK cell activity, and antiviral evaluation of this new series have recently been reported.[156] The parent guanosine analogue **24** and its 3-bromo derivative **25** exhibited the best immunostimulant effect with a maximal enhancement of the NK cytotoxicity by about 7-fold compared to control. Weaker adjuvanticities were measured for the 3-amino and 3-methyl derivatives, **26** and **27**, respectively. Interestingly, the ex vivo determined activation of murine NK cells against T-cell lymphoma (YAC-1) target cells correlated well with the in vivo antiviral activity of members of this series. At 200 mg/kg, the guanosine analogue **24** provided total protection against SFV-induced mortality in mice and 11/12 (92%) of the animals survived when protected by 100 or 200 mg/kg of **25**.[156] At 100 mg/kg, efficacies of **24**, **25**, and 7-thia-8-oxoGuo (**12**) were comparable. At the same dosage, the 3-methyl derivatives **27** protected 8/12 (67%) mice from mortality.[156] The 3-methoxy derivative **28** was very slightly active (17% survivors compared to 8% in the control) and 6-amino-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidin-3,4(2*H*,5*H*)-dione (**29**) was totally inactive in this in vivo antiviral test (data not shown). All these guanosines showed no significant in vitro antiviral activity against Semliki Forest virus.

**Antitumor Potency.** Induction of a specific and potent immune response against tumors is another promising area of therapeutic interest covered by the guanosine deriva-

Table IV. Activity of 7-DeazaGuo 13 on the inhibition of B16-F10 Murine Melanoma Artificial Metastasis in Lungs of C57Black/6J Mice[a]

| assay no. | | no. of mice per assay | harvested lungs from mice (day)[b] | colonies of B16-F10 in lungs: Mean[c] ± SD[d] (Range no. =) |
|---|---|---|---|---|
| 1 | control[e] | 6 | day 17 | 69.00 ± 31.40 (24–112) |
| | treated[e] | 6 | day 17 | 0.14 ± 0.38 (0–1) |
| 2 | control[e] | 7 | day 18–19 | 88.14 ± 23.72 (57–123) |
| | treated[e] | 7 | day 18–19 | 0.17 ± 0.41 (0–1) |

[a] B16-F10 murine melanoma cells were maintained in vitro; cell density was $5 \times 10^5$ mL$^{-1}$ in Hanks' balanced salt solution.[116] Amount of 0.2 mL of cells was injected by iv (tail vein) at day 0 for each mouse. [b] After 17–19 days, the mice were sacrificed and artificial metastasis colonies in the lung for each mouse were counted. [c] Average of B16-F10 colonies in lungs from six or seven mice. [d] Standard deviation. [e] Mice were injected ip with a 2% aqueous sodium bicarbonate solution (control) or with 7-deazaGuo (150 mg/kg) in 2% aqueous sodium bicarbonate in half-daily doses (75 mg/kg bid) on day −1, day 4, and day 9.

tives. Initial studies in mice in 1989 demonstrated that 7-thia-8-oxoGuo could activate macrophages which exhibited potent inhibitory effects against P815 and EL-4 tumor growth in vitro.[143] In fact, 7-thia-8-oxoGuo, given to mice for 48 h at a dose of 88 mg/kg ip, was further shown to enhance lymphocyte-mediated antibody-dependent cellular cytotoxicity against P815 tumor cells.[139] 7-Thia-8-oxoGuo increased life span of mice inoculated with P388 leukemia, M5076 reticulum cell sarcoma, and C26 colon carcinoma.[157]

The most remarkable and documented action of 7-thia-8-oxoGuo concerns its unique potential to be successfully used in the treatment of experimental metastases. As early as 1989, 7-thia-8-oxoGuo was shown to inhibit the establishment of B16 melanoma tumor foci in murine lungs.[158] Pretreatment of mice with a single dose of 100 mg/kg 7-thia-8-oxoGuo 24 h before pulmonary B16-F10 tumor inoculation diminished the number of metastatic foci by 90–96%.[116] If this pretreatment was followed by another injection of 7-thia-8-oxoGuo 72 h after tumor challenge, the number of foci was considerably decreased with a maximum reduction level of 98.7%. This compound was also active against established tumor nodules as it inhibited the growth of 3-, 4-, and 5-day-old metastatic tumors by 92%, 87%, and 75%, respectively.[116] In fact, regression of extrapulmonary as well as pulmonary tumor growth was complete with 7-thia-8-oxoGuo treatment. The antitumor action was shown to be largely dependent on macrophage activation.[116] 7-Deazaguanosine, given ip in half daily doses of 75 mg/kg on days −1, +4, and +9 was also highly active in this same experimental model. The number of B16-F10 colonies in lungs was reduced by 99.8% 17–19 days after tumor challenge (Table IV). In another experiment, intraperitoneal injections of 7-thia-8-oxoGuo 24 h after subcutaneous implantation of reticulum cell sarcoma M5076 fragments in the flank of $BDF_1$ female mice dramatically decreased the number of metastatic foci in liver and lungs.[159] After treatment at the dose of 62 mg/kg, nearly no foci were apparent in the liver, compared to an average number greater than 130 in the control, and their presence was statistically reduced in the lungs. No further metastases were detectable in the kidneys. At a dose of 104 mg/kg, metastatic foci had completely or almost completely disappeared from liver and lungs, respectively.[159]

A series of 2-amino-9-β-D-ribofuranosylpurine-6-sulfenamides, -sulfinamides, and -sulfonamides have been recently synthesized and evaluated for their antitumor effect.[160] One of the most potent compounds, (*R*,*S*)-2-amino-9-β-D-ribofuranosylpurine-6-sulfinamide (sulfinosine, **30**, Chart II) has been shown to stimulate macrophage selectively. Extended in vivo antitumor studies have been performed with this compound. Increase in life span of L1210-inoculated mice was dose-dependent and maximum protection (361% with two cures) was provided by twice daily ip bolus injections (62 mg/kg) given for 7 days following tumor inoculation.[161] Sulfinosine was even shown to be highly active in vivo against L1210 leukemia refractory to many other antitumor agents as well as against a number of different solid tumors like the colon carcinoma C26, the reticulum cell sarcoma M5076 or the human melanoma LOX.[161] Sulfinosine inhibited by 80% the growth rate of two human colon (CX-1) and human mammary (MX-1) carcinoma implanted in mice.[161] Like 7-thia-8-oxoGuo, sulfinosine was also effective in the reduction of metastatic foci following subcutaneous implantation of reticulum cell sarcoma M5076 in mice. Treatment at day 1, 4, and 7 with 104 mg/kg sulfinosine considerably decreased the number of tumor nodules in liver, lung, or kidney, and total clearance of metastatic foci was manifest with regimens of 173 mg/kg sulfinosine.[159]

Development of metastases in different parts of the body is a major cause of death after failure of a primary tumor treatment. Optimal therapy should afford eradication of the last metastatic cells in order to restore the physiological balance between cell proliferation, cell differentiation, and cell death within each tissue. In this perspective, many studies have concerned the direct administration of natural or recombinant cytokines or antitumor peptidic factors which are natural stimulators of the immune response. Guanosine derivatives and analogues are low molecular weight immunopotentiators which activate various aspects of the immune response, depending on the guanosine nucleoside structure. Good protection against C26 colon carcinoma mortality in mice can be readily provided by cotreatment with divided low doses of sulfinosine and 7-thia-8-oxoGuo.[159] Increases of murine life span as high as 210%, with existence of a long-term survivor considered as cured, were obtained with regimens of these two nucleosides at doses as low as 62 mg/kg given twice a day on day 1, 4, and 7 for sulfinosine and 37 mg/kg given once a day on day 1, 5, 9, 13, 17, and 21 for 7-thia-8-oxoGuo.[159] The longer treatment over 21 days with 7-thia-8-oxoGuo allowed recruitment of natural killer cells and activation of B-cell immunity which resulted in synergy of action and contributed to enhanced antitumor effect.

The concept of a cancer vaccine has recently regained some interest.[5,161–165] Vaccine against human hepatitis B is available in many countries, and systematic infant immunization is hoped to greatly reduce primary hepatocellular carcinoma by preventing the establishment of the chronic carrier state of the virus.[5,166] When cancers are not from viral origin, the basic concept underlying the use of a tumor vaccine for treatment or prevention corresponds to the hypothesis that immunizing patients with cell-surface tumor-associated antigens would enhance the immunological response to neoplasia.[5,167,168] Unfor-

tunately, most of the antigens expressed on tumor cell surface are only weakly immunogenic. 7-Thia-8-oxoGuo has recently been successfully used as an adjuvant to increase the efficacy and potency of a tumor vaccine.[169] 7-Thia-8-oxoGuo highly enhanced the protection conferred to mice challenged with live L1210 leukemia cells after having been vaccinated with irradiated L1210 leukemia cells. When administered 2 and 4 h after each vaccination, 7-thia-8-oxoGuo was able to potentiate the survival time of treated mice by a maximum of 15-fold over the control and to greatly increase their survival rate to 81% compared to 31% in the group of mice that received the vaccine only.[169] The adjuvanticity offered by immunostimulators like guanosine derivatives and analogues should be carefully considered in such an approach for the prevention and treatment of cancer.

Very recently, phase I clinical trials have been conducted with loxoribine at the University of Pittsburgh.[145] Sixteen patients with advanced cancer have been treated with doses of 1, 5, and 10 mg/kg. Preliminary results show these two lower doses of loxoribine did not induce major signs of toxicity, confirming the innocuousness observed in animals at doses up to 160 mg/kg. Initial pharmacokinetic, immunologic, and therapeutic data are eagerly awaited.

## Mechanism of Action of Guanosine Nucleosides in Activation of the Immune Response

Unlike many classical components of the immune system, guanosine derivatives and analogues do not exert their activity by binding to the cell surface membrane. Goodman and Weigle[170] showed in 1984 that 8-BrGuo was readily transported across the cell membrane. Intracellularly, two exchangeable pools, one rapid ($t_{1/2}$ = 4.5 min) and one slow ($t_{1/2}$ = 275 min), as well as the existence of good correlations between binding affinity, ligand specificity, and nucleoside activity were further demonstrated on murine CBA/CaJ B lymphocytes.[171] In fact, within the murine B cell, the guanosine derivatives do remain in the cytoplasm but are rapidly concentrated in the nucleus. Studies with 8-BrGuo indicated this nucleoside was not phosphorylated by B lymphocytes and was not incorporated into nucleic acid.[172] 7-*n*-Propyl-8-oxoguanosine (11), reported to demonstrate an immunobiological activity comparable to that of 7-Me-8-oxoGuo,[113] was recently shown to bind in its unmetabolized form to a nuclear component of murine B lymphocytes.[173] This binding site, which exhibited an apparent molecular mass of 30–40 kDa, has appeared to possess all the characteristics of a receptor site.[173] Nuclear binding was specific, saturable, with a high affinity $K_d$ constant of ~7 μM. This binding was determined to be maximal after only 30 min of B-cell incubation with 11.[173] Ligand specificity was demonstrated by binding inhibition studies with other guanosine analogues such as loxiribine 8, 8-BrGuo (4), 8-bromoguanine, and 6-thioguanosine. Inhibition data were in agreement with the immunomodulating activities of these various nucleosides as 100 μM of 8 was sufficient to completely abrogate the binding of 7 μM [$^3$H]7-*n*-propyl-8-oxoguanosine, and conversely, the same concentration of 6-thioguanosine, which is devoid of immunostimulatory activity, was totally ineffective in binding inhibition.[173] Murine splenic CBA/CaJ B cells were shown to express approximately $2 \times 10^4$ high affinity binding sites per cell.[173] However, saturable binding was not demonstrable for cells which do not respond functionally to nucleosides such as thymic lymphocytes.[173] Both a rapid and a slow exchangeable pool of 11 coexisted within murine B lymphocytes, and Goodman suggested the low affinity ($K_d \approx$ 500 μM) and the high affinity binding sites displayed selective roles in antigen-independent inductive and antigen-dependent differentiative events, respectively.[113]

Other important features of the mechanism of action of these immunomodulators are available from a study by Goodman et al.[174] concerning the interaction of 7-Me-8-oxoGuo with some classical biochemical pathways in murine splenic B lymphocytes. Within the first hour, interaction with GTP binding proteins or modulation of phosphoinositide metabolism were not involved in the mechanism of action, nor was a modification of the intracellular free calcium concentration during the first 7 min. Likewise, activity of 7-Me-8-oxoGuo appeared to be independent of protein kinase C (PKC) activity for the first 10 min.[174] These results are not to be interpreted as an exclusion of such biochemical pathways from the overall mechanism of action of these immunomodulators. They only demonstrate that the early events of the interaction of 7-Me-8-oxoGuo with B cells are independent of G proteins, intracellular free calcium, inositol phosphates, and PKC. In fact, when these pathways are analyzed after a longer period of time, on other cells of the immune system, results are quite different. In murine peritoneal exudate cells, pertussis toxin, which uncouples receptors from G proteins to annul signal transduction, has been shown to inhibit the phagocyte-activating effect of 8-BrGuo and 7-thia-8-oxoGuo.[175] Furthermore, in murine spleen cells, inositol 1,4,5-triphosphate and diacylglycerol levels were greatly increased by a 48–72 h incubation with 7-thia-8-oxoGuo.[176] A biphasic increase in both the cytosolic and particulate PKC activity were also determined, with a maximal and greater enhancement of the cytosolic fraction by 48 h.[176] Thus, these pathways appear to be highly activated when these guanosine nucleosides are added to leukocyte cultures over a period of time sufficient to activate the genetic expression of the immunomodulator activity. However, a specific activation of murine B cells has been shown to possibly occur independently of any direct PKC activation. Mond showed 8-SHGuo could restore antiimmunoglobulin-stimulated B-cell proliferation in PKC depleted cells.[177] Under these conditions and in association with the induction of mitogenesis, an enhancement of the synthesis of a nuclear protein, numatrin, was observed.[178] From these studies it is clear that stimulation by the guanosine derivatives of the B-cell antigen-dependent differentiative process can be independent of PKC phosphorylation pathways but that normal and complete activation of the immune system implies their participation.

B-cell activation is not the only aspect to be considered. Activation of the cytotoxic cells, killer cells and macrophages, of the immune system has appeared to be fundamental to the action of these guanosine derivatives against solid tumor metastases.[116,179,180] Abrogation of the cytotoxic effect of killer cells by administration of anti-asialo-$GM_1$ antibody after treatment with 7-thia-8-oxoGuo resulted in a complete loss of the antimetastatic activity of the nucleoside against pulmonary B16 melanoma in C57BL/6 mice.[116] Although 100 mg/kg 7-thia-8-oxoGuo, when administered ip 24 h before tumor inoculation, was able to reduce the number of B16 tumor foci in the lungs from a mean of 125 ± 17 to a mean of 13 ± 0.9, administration of anti-asialo-$GM_1$ antibody ip 2 h after

7-thia-8-oxoGuo raised this number to a value greater than 200.[116] Likewise, when macrophages were inhibited by administration of carrageenan or silica to mice 5 h before 7-thia-8-oxoGuo, the protective effect of the nucleoside against metastatic B16 tumor growth was significantly affected as the number of tumor foci in the murine lungs was enhanced to a mean of 58 ± 11.[116]

Although the role of killer cells and macrophages is evident in the antimetastatic activity of 7-thia-8-oxoGuo, their role in the antiviral activity of the guanosine derivatives and analogues seems less crucial. NK cells are well known to exert cytotoxic effects against cells infected with many viruses.[181] However elimination of NK cells with anti asialo-$GM_1$ antibody or conversely, supplementation with activated NK cells, did not appear to affect in any way the protection conferred by 7-thia-8-oxoGuo to SFV-infected mice.[144] NK cell-deficient beige mice were also shown to be protected to some extent from SFV mortality by 7-thia-8-oxoGuo.[144] Furthermore, 7-thia-8-oxoGuo was still effective against SFV infection in mice even after inactivation of the macrophages by treatment with fumed silica.[144]

The antiviral activity of the guanosine derivatives and analogues appears to be primarily due to the induction of IFN. 7-Thia-8-oxoGuo[182] and 7-deazaGuo[115] have been found to be potent inducers of IFN secretion in mice. IFN serum titers increased 10 times higher with 7-deazaGuo than with 7-thia-8-oxoGuo.[115] In mice treated with 7-deazaGuo, induction was determined to be rapid with high levels (>$10^5$ units/mL) detected only 1 h after ip injection.[115] Oral administration slightly delayed the occurrence of the IFN peak titer an additional hour.[115] IFN levels remained stable for 1–4 h and then declined between 4 and 8 h after treatment.

The role of IFNs as antiviral agents is well documented.[183,184] Antiviral protection provided by guanosine derivatives and analogues seems to be specifically conferred by IFN$\alpha$, as demonstrated by studies with anti-IFN antibodies.[144] Although treatment of Swiss Webster mice with 10 000 units IFN$\alpha/\beta$ given 24, 18, and 2 h before SFV inoculation has proved to be less efficient (75% survivors) than 100 mg/kg per day of 7-thia-8-oxoGuo given 24 and 18 h before virus challenge (92% survivors), this protective effect of 7-thia-8-oxoGuo was significantly reduced to 42% survivors by cotreatment with 1000 units of anti-IFN$\alpha/\beta$ antibody given 23, 17, and 2 h before virus inoculation.[144] In the same experiment, cotreatment with identical doses of anti-IFN$\beta$ or $\gamma$ antibodies did not interfere with 7-thia-8-oxoGuo antiviral effect as the number of survivors was maintained at 83% with anti-IFN$\beta$ and 92% with anti-IFN$\gamma$.[144] The abrogative effect of anti-IFN$\alpha$ antibody was further demonstrated on SFV-treated mice by complete obliteration (0% survivors) of the protective effect (92% and 83% survivors, respectively) of 25 or 50 mg/kg per day of 7-thia-8-oxoGuo, given 24 and 18 h before SFV inoculation, by cotreatment 30 min after each 7-thia-8-oxoGuo injection with 2000 units of anti-IFN$\alpha/\beta$.[144] Likewise, similar treatment with anti-IFN$\alpha/\beta$ antibody was shown to totally abrogate (0% survivor) the anti-Punta Toro virus therapeutic activity (80% and 100% survivors, respectively) of 25 or 50 mg/kg per day of 7-thia-8-oxoGuo given 24 and 31 h after virus inoculation.[152]

Protection afforded by IFN can be direct, by lysis of the virus-infected cells, or indirect, by IFN activation of macrophages and killer cells. Against viral infection, such activation of macrophages does appear however to participate in the total response of the immune system. In in vitro studies, anti-IFN$\alpha/\beta$ and anti-IFN$\gamma$ antibodies were shown to decrease the 8-BrGuo elicited activity of macrophages and NK cells against mastocytoma P815 cells.[133]

Guanosine derivatives and analogues not only induce IFN production but also stimulate the secretion of other cytokines both in vitro and in vivo. 8-BrGuo has been reported to induce IL-1 activity from both murine splenic adherent cells and the macrophage cell line $P388D_1$.[134] Supernatants of these 8-BrGuo-treated cells were mitogenic for murine thymocytes and costimulated purified B cells in the presence of anti-$\mu$ antibodies. This activity was abrogated by anti-IL-1 antibodies. Similar results have been found with 7-thia-8-oxoGuo and the IL-1 induced production has been confirmed by radioimmunoassay experiments.[185] Although 7-thia-8-oxoGuo elicited an IL-2-like activity in the supernatant of murine B-cell cultures,[176] this nucleoside was further shown to be unable to induce IL-2 in cell culture and in serum of treated mice or to cause an increase in IL-2 receptors in human peripheral blood mononuclear cell cultures.[185] 7-Thia-8-oxoGuo has been recently found to induce IL-6 production in murine spleen cells, human peripheral blood mononuclear cells, and the human monocytic leukemia cell line THP-1.[186] Stimulation of macrophages would be expected to result in secretion of tumor necrosis factor (TNF).

Such cytokines play fundamental roles in many specific aspects of the immunostimulant effects of the various guanosine analogues. Indeed, it would now appear that specific guanosines are capable of inducing highly defined cytokine gene expression in leukocyte cells. TNF has been shown to be able to induce overexpression of major histocompatibility complex (MHC) class I antigens on the surface of different human tumor cells.[187] This overexpression was further shown to induce higher immunogenicity of these cells.[187] Unlike untreated control cells, TNF$\alpha$-treated breast carcinoma or ovarian adenocarcinoma cells were able to stimulate autologous and allogeneic blood lymphocytes and to generate autotumor cytotoxic cells.[187] Sharma et al.[169] have suggested that this property could explain the above discussed potentiation of the efficacy of a tumor vaccine against L1210 leukemia offered by treatment of mice with 7-thia-8-oxoGuo. Since 1987, clinical trials have been underway to determine the utility of TNF in the treatment of different types of human cancer.[7,188] TNF also exhibits antiviral properties which can be synergistic with IFNs in vitro.[189,190] IL-6 acts on activated B cells to induce IgM, IgG, and IgA secretion.[191-193] IL-6 induces T-cell growth and differentiation[194] and augments myeloma and hybridoma growth.[195,196] IL-6 enhances the activity of human NK cells[197] and the generation of cytotoxic T cells from murine thymocytes[198,199] as well as in both the murine and human allogeneic mixed leukocyte reaction.[200] It has been determined to exert antitumor effects on many cell lines in vitro[201,202] as well as on some murine tumor models in vivo.[203,204] IL-1 and IL-6 together enhance the accessory activity of human mature macrophages, which leads to stimulation of lymphocyte proliferation.[205] Recently IL-1 has been shown to synergize with IFN$\alpha$ to augment its effect against metastatic Friend erythroleukemia cells in mice.[206] Although these cytokines exhibit interesting activities for the treatment of viral infections and cancer,

many problems limit their direct use in clinical practice.[207] Since they are rapidly metabolized, therapeutic responses have proved relatively short-lived due to enzymatic degradation. The administration of cytokines directly in the bloodstream can affect many tissues and cause undesired adverse effects. Recently, TNF degradation products have been implicated in causing such toxicity.[209]

The development of low molecular weight immunomodulators, such as the above described modified guanosines, which are metabolically more stable and able to selectively induce leukocytes to generate specific cytokines in situ, appears to deserve further consideration in the employment of desired cytokine immunotherapy.

## Modulation of Cytokine Gene Expression

IFN-$\alpha$-, IL-1-, IL-6-, and TNF-induced production can largely account, directly or indirectly, in part for the observed antiviral and antitumor activity of certain guanosine nucleosides described here. Nevertheless, relatively little is known about the mechanism of how these guanosine derivatives and analogues cause cytokine expression. Although IFN-$\alpha$ is synergistic with 8-BrGuo to enhance B-cell response to antigen, studies using specific antibodies have demonstrated IFN-$\alpha$ was not involved in 8-BrGuo-induced potentiation of the immune response in B cells.[132] Thus, IFN-$\alpha$ is unlikely to play a primary role in the mechanism of action of guanosine derivatives in these cells. IFN-$\alpha$ or -$\beta$ (type I IFN) gene expression can be modulated by the transcriptional activator IRF-1 and the repressor IRF-2.[209-211] Regulation of IFN-$\beta$ by IRF-1 involves activation of the nuclear factor $\kappa$B (NF-$\kappa$B).[212-214] However IFN-$\alpha$ gene family is not regulated by NF-$\kappa$B.[213-215] Increase in INF-$\alpha$ mRNA can occur via stimulation of IRF-1 by IL-1 and TNF cytokines.[216] It is thus likely that enhancement of the IFN expression by guanosine derivatives and analogues is secondary to an induced primary expression of IL-1 and TNF.

In vitro[108,123,124,128] and in vivo[150,152] studies have shown that certain guanosine derivatives and analogues are effective in their stimulation of the immune response, even when added late to the cell culture or late in the animal. Although many factors can modulate B-cell differentiation, IL-6 has been shown to be absolutely required for antibody production.[192] Similar to guanosine nucleosides, IL-6 induces differentiation in unstimulated B cells and has been determined to be a late-acting factor, since anti-IL-6 antibodies were able to inhibit the cytokine activity even when added on the fourth day of an 8-day B-cell culture.[192]

The three above-mentioned cytokines, IL-1, IL-6, and TNF, have been determined, as reported above, to be induced by certain guanosine derivatives and analogues. A common nuclear transcription factor, NF-IL6, might be involved in the regulation of these cytokines.[217] Originally, this DNA-binding protein was identified as a nuclear factor responsible for IL-6 expression.[218] NF-IL6 binding consensus sequences have been recently identified in the promoter regions of IL-1, IL-6, and TNF.[217-219] NF-IL6 has been cloned and found to belong to the C/EBP leucine zipper family.[218] Recently, another member of this family, NF-IL6$\beta$, has been cloned, characterized, and reported to be very similar to NF-IL6, although its induction was shown to occur less readily.[220] To date, regulation of the gene expression of these two nuclear transcription factors remains poorly understood. However, NF-IL6 activity has been shown to be regulated both at the transcriptional and the posttranscriptional levels.[218,219] NF-IL6 mRNA and protein are expressed constitutively in many cell lines, and their levels can be further increased after external stimulation by several agents like lipopolysaccharide or inflammatory cytokines.[218] Induction of NF-IL6 activity by IL-1 appears to proceed most likely from a posttranscriptional modification of an already produced inactive form of NF-IL6.[218] A similar pathway can be proposed for the induction of specific cytokine production by certain guanosine derivatives. However, since no synergy has been found between such nucleosides and IL-1 in B cells,[132] signals provided by these two stimulating factors must be slightly different and the triggered cellular target(s) leading to cytokine gene expression distinctly specific. The manner by which IL-1 initiates NF-IL6 activity has not yet been elucidated but one can propose a mechanism of action for the guanosine-type immunomodulators which correlates with the various findings available to date. Recent studies have demonstrated that cAMP is capable of stimulating rNF-IL6, the rat homolog of NF-IL6, to undergo phosphorylation and to translocate from the cytoplasm to the nucleus.[221] Studies on another nuclear factor, NF-$\kappa$B, by Baeuerle and Baltimore,[222] have clearly demonstrated compartmentation of transcription factors may play important roles in the control of gene expression. In fact, once translocated in the nucleus, rNF-IL6 was able to bind to DNA and to induce transcriptional activity.[221] Thus, a mechanism controlling NF-IL6 (or NF-IL6 related factors) regulation appears to be quite similar to one described for NF-$\kappa$B,[223,224] and shift of the functional form of the nuclear factor from the cytoplasm to the nucleus has important functional consequences. In this context, the increase in synthesis of numatrin, reported to occur within 1–2 h following 8-SHGuo interaction with B cell cultures,[225] could be of fundamental importance in the mechanism of action of certain guanosine derivatives. Numatrin is a nuclear protein which appears to be identical to the nucleolar protein B23,[178] a major shuttle protein between nucleus and cytoplasm,[226] which has been shown to interact with nucleic acids.[227,228] Numatrin has been found to be associated with the proliferation of B lymphocytes in mice.[225,229] Interestingly, numatrin has the same $M_r$ (40 kDa)[225] as the protein to which guanosine nucleosides have been reported to specifically and tightly bind in the nucleus.[175] This shuttle protein could be involved in the translocation of a functional form of NF-IL6, or of a related transcription factor, from the cytoplasm to the nucleus of leukocytes where it could stimulate specific cytokine gene expression and therefore, induce overall the observed potentiation of the immune system.

## Conclusion

Modulation of the immune system can be provided by adenosine and guanosine derivatives. Adenosine derivatives such as 2-CdA (**1**) or fludarabine (**2**) induce apoptosis in leukocyte and cause breakdown of the immune response. On the other hand, many guanosine derivatives induce leukocyte proliferation and/or differentiation that lead to an unique potentiation of the immunity. Both mechanisms of action are much likely to involve a modulation of leukocyte genetic expression and a common pathway might be modulation of cytokine gene expression. IL-6 plays an important role within the cytokine network[230-232] and regulation of IL-6 expression might be essential to the activity of both adenosine and guanosine immunomodulators. Such a role might in fact involve the numatrin/

B23 shuttle protein. The exact role of this major shuttle protein, migrating constantly back and forth between nucleus and cytoplasm, is still unclear, but it is likely to be involved in the cytoplasmic regulation of nuclear activities, since nuclear factor such as NF-IL6 or ubiquitous NF-κB have to translocate through the nuclear membrane to be able to activate specific genetic expression. Such a direct modulation in leukocytes of nuclear activity by adenosine and guanosine immunomodulators, leading to a modulation of leukocyte genetic expression and to a consequent modulation of the immune system, surely deserves further consideration.

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