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Neuramide inhibits Epstein-Barr virus-induced transformation activity and proliferation of transformed B lymphocytes

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Abstract

Neuramide, in Italy, is a prescription drug and contains various polypeptides, with molecular weights ranging between 10 000 and 1000. It is commonly used in Italy for the clinical therapy of varicella-zoster and other viral herpetic diseases. In the present study, we investigated its effect on the in vitro transformation activity and transformed status of the Epstein-Barr virus (EBV), an herpesvirus closely associated with infectious mononucleosis (IM), nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL). Antiviral effects of neuramide were evaluated on viral-induced immortalization, transformed status and DNA synthesis. Our results show that simultaneous and/or post-treatment of EBV-infected lymphocytes with neuramide, at a concentration of 2 inhibiting units (IU)/ml, blocks EBV-mediated transformation and related events, whereas pretreatment does not show any inhibiting activity against EBV. Neuramide was also found to be a potent inhibitor of the proliferation of EBV-transformed B lymphocytes in vitro. These results suggest that the molecular mechanisms of action of the drug should be investigated more closely and also support the necessity to further purify the active polypeptides, in order to improve its possible in vivo efficacy in EBV-associated diseases, such as infectious mononucleosis and other lymphoproliferative diseases.

Keywords: Antiviral agent; Epstein-Barr virus; Lymphoproliferative disorders; Neuramide; Transformation

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1. Introduction

Neuramide is a prescription drug (Difco Cooper SpA, Varese, Italy) commonly used in Italy in clinical practice for the therapy of herpes zoster, genital herpes, herpetic keratitis, neuritis and varicella (Italian Health Department authorization code no. 005104029). Chemically, it is a pool of different polypeptide fractions extracted from the gastric mucosa of pig, with a molecular weight ranging between 10 000 and 1000. It has been shown to have antiviral and immunostimulant effects (Baron and McKerlie, 1981; Alberini et al., 1982; Baron and Albrecht, 1982). A number of clinical studies have also shown its in vivo effectiveness in the treatment of varicella (Kumar and Baron, 1981; Bruni et al., 1984; Baron et al., 1989), herpes zoster (Kumar and Baron, 1981; Kumar et al., 1984; Baron et al., 1989) and herpetic stomatitis (Kumar and Baron, 1981; Kumar et al., 1984). Furthermore, some investigators have demonstrated an in vitro inhibiting effect on varicella-zoster virus (VZV) replication (Albrecht et al., 1983). It appears that neuramide acts in a dose-dependent manner at a point after virus adsorption, but prior to VZV-directed DNA synthesis. Neuramide-sensitive replication events apparently continue into late stages of VZV replication (Albrecht et al., 1983).

Kinetic studies have revealed that neuramide works in a similar fashion against other viruses, such as influenza A virus (Antonelli et al., 1986). In fact, the maximum inhibiting effect was observed up to 5 h after virus penetration. This and other studies performed by different techniques revealed that the antiviral activity is contained in a low molecular weight fraction (MW = 1000) (Antonelli et al., 1986; Miraglia et al., 1990).

Since one of the drugs most effective against Epstein-Barr virus (EBV) infection, acyclovir, is not effective in preventing EBV transformation (Sixbey and Pagano, 1985), and relatively few studies have successfully reported agents able to inhibit EBV-induced growth transformation (Garner et al., 1984; Nemerow and Cooper, 1984; Lin et al., 1989; Cirone et al., 1990), we set up the present study to evaluate the effects of neuramide on EBV-induced immortalization of human B lymphocytes and on viral DNA synthesis, and of its antiproliferative effects on EBV-positive Burkitt's lymphoma (BL) cell lines.

We have observed that neuramide specifically inhibits B-cell growth transformation and DNA synthesis induced by EBV. We have also observed that both simultaneous and postponed treatments of the target cells with neuramide (2 U/ml) inhibit the EBV-induced growth transformation and all related events. In contrast, pretreatment of EBV-infected lymphocyte cultures with neuramide did not hamper the cellular transformation. Furthermore, our data on BL cell lines show a very strong inhibiting effect on the proliferation of already transformed B cells, also suggesting immunomodulant properties of some fractions of the drug (Baron and McKerlie, 1981; Alberini et al., 1982; Baron and Albrecht, 1982), which may cooperate with the antiviral effects of other fractions. Thus, neuramide could eventually have a therapeutic role in vivo because of its specific effect on EBV de novo transformation and EBV-transformed status.

2. Materials and methods

2.1. Compounds

Neuramide was obtained from Difco Cooper SpA, Varese, Italy, in 1.3- and 3-ml ampules containing 32 inhibiting units (IU) 50%, according to the Baron–McKerlie method: at 1:32 dilution, the drug inhibits by 50% the formation of viral plaques (Baron and McKerlie, 1981). Neuramide has been used at standard dilutions of 1 and 2 U/ml in RPMI 1640 (Flow Irvine, Scotland, UK) according to Albrecht et al. (1983). Such concentrations were effective against other herpes viruses, but not toxic for cell lines or human lymphocytes (Albrecht et al., 1983). Trypan blue (0.4%) stain and phosphate-buffered saline (PBS) were obtained from Gibco (New York). [*Methyl*-^{3}H]thymidine (120 Ci/mmol; 1 mCi/ml) was obtained from NEN.

2.2. Cells and virus

All the cell lines and human lymphocytes were grown in RPMI 1640, 10% glutamine, 20% fetal calf serum (inactivated for 30 min at 57°C), 5% penicillin–streptomycin (5000 IU each).

Cord blood lymphocytes (HUCL) obtained from healthy newborns, were isolated from total blood by centrifugation on density gradient Ficoll-Hypaque, followed by a 2-h incubation at 37°C in tissue-culture flasks to separate adherent cells (macrophages) from non-adherent cells (lymphocytes). B subpopulations have been separated by rosetting with sheep red blood cells and separated by centrifugation on density-gradient Ficoll-Hypaque. HUCL were diluted at a concentration of 2×10^6 cells/ml, then plated at 2×10^5 cells/100 μl per well.

The transforming B95.8 strain of EBV has been isolated from cultures of B95.8 cells, kindly provided to our laboratory by Dr D.V. Ablashi (Bethesda, MD), purified by cesium chloride banding and concentrated 200 $\times$, with an effective transforming titer of 10^{-8} (dilution factor) and used at concentrations from 10^{-1} to 10^{-6} (dilution factor: $\log_2$).

2.3. Immunofluorescence assays

EBV nuclear antigen (EBNA) has been assessed by indirect anti-complement immunofluorescence as previously described (Reedman and Klein, 1973). Briefly, after fixation and permeabilization in cold acetone/methanol (50%:50%), the cells were incubated for 45 min at 37°C with C_3- fraction of the human complement (diluted 1:20) from Cappel (Durham, NC), washed three times with PBS (Gibco, Gaithersburg, MD), followed by incubation under the same conditions with goat IgG antihuman C_3 from Cappel.

2.4. Transformation assays

B-cell-enriched target cells (HUCL) were plated in 96-well flat-bottom plates at 2×10^5 cells/well and resuspended to a final volume of 200 μl. Medium was changed every 3 days. As controls, untreated infected cells, and uninfected cells treated with/without neuramide were included. All tests were done in quadruplicate and repeated for at least three healthy EBV-negative donors. To evaluate virus-induced transformation, lymphocytes were cultivated for a 3-month period to observe the morphological changes associated with transformation, such as the increasing number of cell clumps, EBNA positivity and capability to grow indefinitely in culture. EBNA evaluations were performed at 24 h, and at days 21 and 90 after infection. Pretreatment with neuramide consisted of continuously treating target cells for 24 h prior to infection with neuramide at 1 and 2 IU/ml, washing three times with medium, then infecting with EBV B95.8 strain at different dilutions. In the simultaneous treatment procedure, lymphocytes were exposed to the virus and to neuramide at the same time; neuramide was removed 3 or 9 days after the infection. Post-treatment was done by treating lymphocytes for 3 days with neuramide, at 12 or 24 h after infection with EBV, not removing the virus from the culture medium.

2.5. DNA synthesis assays

DNA synthesis was evaluated by [*methyl*-^{3}H]-thymidine incorporation: 10^5 cells/well in 200 μl of the proper medium were plated in round-bottom 96-well plates. On days 3 and 6 after treatment, cells were labeled with 1 μCi of [*methyl*-^{3}H]thymidine (120 Ci/mmol) and incubated for 4 h. Cells were then harvested onto glass fiber filters using an automatic cell harvester. Filters were then washed twice with 2 and 4 ml of cold PBS, 2 and 4 ml of 10% cold trichloroacetic acid, then twice with 4 ml cold 5% trichloroacetic acid and 1 ml cold ethanol. Incorporation was expressed in counts per min (cpm) and measured in a β-counter with scintillation liquid. The arithmetic mean of the tests made in triplicate was calculated; the variation among each triplicate was not meaningful ($< 10\%$).

2.6. Antiproliferative assays

The transforming strain B95.8 of EBV induces: (i) EBNA within 6–20 h after infection (Reedman

and Klein, 1973); (ii) DNA synthesis; and (iii) B-lymphocytes immortalization, resulting in indefinite growth in vitro. To distinguish the antiviral properties of the neuramide preparations from their cytotoxic effects on EBV-transformed cells, we calculated the 50% cytostatic concentration (CC_{50}) expressed in IU/ml, and the rate of mortality (with 2 IU/ml neuramide) expressed in percentage. The cells (2×10^5 cells/well) were seeded in a 24-well tissue-culture plate with the different dilutions of the test compound, and further incubated at 37°C. Both EBV-positive and EBV-negative transformed B lymphocytes, as well as normal and EBV-immortalized B lymphocytes and other lymphoid cell lines (non-B) were used as controls. Neuramide was used at increasing final concentrations from 0.2 to 6.0 IU/ml. After 3 days, the number of viable cells was determined in a Neubauer cell-counting chamber by trypan blue staining. The cell death rate at 2 IU/ml of neuramide was calculated after subtraction of the cell death values of the untreated controls. The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration required to reduce by 50% the number of viable cells. Results are the arithmetical mean of the values of the experiments performed in quadruplicate.

3. Results

3.1. Effect of the simultaneous treatment of neuramide on EBV transformation activity and on cellular DNA synthesis

3.1.1. Effect on transformation

We first investigated the effect of neuramide on EBV transformation of HUCL and on induction of cellular DNA synthesis (Table 1). Initially, we pretreated the cells prior to EBV infection; however, no inhibiting effect was observed. Subsequently, we attempted to treat cells with neuramide at the time of infection and observed a powerful antiviral effect (83–100%) when 2 IU/ml of neuramide was used. However, we were also able to detect partial antiviral activity at lower concentrations of the drug. An optimum inhibiting effect was observed when the cells were treated for 3 days; longer treatments did not improve the blocking effect.

3.1.2. Effect on DNA synthesis

To confirm our observations on the blocking effect of the EBV transformation of HUCL by neuramide, we assayed the effect of neuramide on DNA synthesis. In fact, the transforming strain of EBV B95.8, has been shown to induce viral and host DNA synthesis. The HUCL cells were infected with various titers of virus (10^{-1}–10^{-6}) and treated with neuramide according to the same protocols used for the transformation assays. The results in Table 1 show that 2 U/ml of neuramide given at the time of virus infection strongly inhibit (73–77%) the increase in DNA synthesis stimulated by the virus in cells infected with 10^{-1} or 10^{-2} titered virus. When the cells were infected at lower titered virus (10^{-6}), an almost complete inhibition was observed (96.3%).

3.2. Effect of post-treatments at 12 and 24 h with neuramide on EBV transformation activity and on cellular DNA synthesis

3.2.1. Effect on transformation

In this set of experiments, we tested the blocking effect of neuramide at 12 h and 24 h post-infection to assess if this antiviral blocking activity could be due to a direct interference of neuramide on EBV or after the virus penetration into the cell. Due to the poor antiviral effectiveness shown by neuramide at 1 U/ml in the simultaneous treatment experiments, we performed post-treatment experiments with this drug only at 2 U/ml concentration. No inhibiting effect of neuramide in HUCL cells infected with EBV at a titer of 10^{-1} following the 12- and 24-h post-treatment was observed. However, when cells were infected with 10^{-3} titered virus, complete blockage could be observed with the 24-h post-treatment (Table 2).

3.2.2. Effect on DNA synthesis

Post-treatment of neuramide (Table 2) at 12 and 24 h after infection resulted also in a complete inhibition of DNA synthesis. The block in DNA synthesis is in agreement with our transfor-

Table 1
Effect of neuramide treatment on EBV-induced transformation and DNA synthesis of HUCL infected with B95.8 EBV

Treatment[a]	Viral dilution	Appearance of transformation (days postinfection)	No. transformed/no. attempted	% Inhibition	% Inhibition of [^{3}H]thymidine incorporation[b]
Medium control	None	NT	0/12	—	0
Neuramide (1 U/ml)	None	NT	0/12	—	N/A
Neuramide (2 U/ml)	None	NT	0/12	—	10.2
None	EBV 10^{-1}	12	12/12	—	—
Neuramide (1 U/ml)	EBV 10^{-1}	12	12/12	0	N/A
Neuramide (2 U/ml)	EBV 10^{-1}	18	2/12	83.3	73.6
None	EBV 10^{-2}	12	12/12	—	—
Neuramide (1 U/ml)	EBV 10^{-2}	12	12/12	0	N/A
Neuramide (2 U/ml)	EBV 10^{-2}	18	1/12	91.6	77.0
None	EBV 10^{-3}	12	12/12	—	—
Neuramide (1 U/ml)	EBV 10^{-3}	18	12/12	0	N/A
Neuramide (2 U/ml)	EBV 10^{-3}	NT	0/12	100	62.4
None	EBV 10^{-4}	12	11/12	—	—
Neuramide (1 U/ml)	EBV 10^{-4}	21	7/12	36.3	N/A
Neuramide (2 U/ml)	EBV 10^{-4}	NT	0/12	100	65.2
None	EBV 10^{-5}	12	6/12	—	—
Neuramide (1 U/ml)	EBV 10^{-5}	18	1/12	83.3	N/A
Neuramide (2 U/ml)	EBV 10^{-5}	NT	0/12	100	62.7
None	EBV 10^{-6}	12	3/12	—	—
Neuramide (1 U/ml)	EBV 10^{-6}	NT	0/12	100	N/A
Neuramide (2 U/ml)	EBV 10^{-6}	NT	0/12	100	96.3

U, unit; NT, non-transformed.
[a] Neuramide treatment was performed for 72 h and included at the time of infection.
[b] Measured by cpm after 72 h.

mation results, further suggesting that neuramide is able to inhibit EBV transformation and related events. [^{3}H]thymidine uptake in infected HUCL cells at all different viral titers was reduced to the values of the negative controls. Furthermore, the effects did not seem to be due to a toxic effect of neuramide on HUCL, since uninfected HUCL treated with neuramide still incorporated the same amount of [^{3}H]thymidine as cells not exposed to the drug. This set of tests on DNA synthesis fully

Table 2
Effect of post-treatment with neuramide on EBV-induced transformation and DNA synthesis of HUCL after infection with B95.8 EBV

Treatment[a]	Viral dilution	Appearance of transformation (days postinfection)	No. transformed/no. attempted	% Inhibition	% Inhibition of [^{3}H]thymidine incorporation[b]
Medium control	None	NT	0/12	—	—
Drug control	None	NT	0/12	—	0
None	EBV 10^{-1}	7	12/12	—	—
Neuramide (2 U/ml, 12 h)	EBV 10^{-1}	14	12/12	0	100
Neuramide (2 U/ml, 24 h)	EBV 10^{-1}	14	12/12	0	100
None	EBV 10^{-2}	7	12/12	—	—
Neuramide (2 U/ml, 12 h)	EBV 10^{-2}	21	4/12	60	100
Neuramide (2 U/ml, 24 h)	EBV 10^{-2}	21	6/12	50	100
None	EBV 10^{-3}	14	12/12	—	—
Neuramide (2 U/ml, 12 h)	EBV 10^{-3}	21	3/12	75	100
Neuramide (2 U/ml, 24 h)	EBV 10^{-3}	NT	0/12	100	100
None	EBV 10^{-4}	21	11/12	—	—
Neuramide (2 U/ml, 12 h)	EBV 10^{-4}	NT	0/12	100	100
Neuramide (2 U/ml, 24 h)	EBV 10^{-4}	NT	0/12	100	100
None	EBV 10^{-5}	21	8/12	—	—
Neuramide (2 U/ml, 12 h)	EBV 10^{-5}	28	1/12	87.5	100
Neuramide (2 U/ml, 24 h)	EBV 10^{-5}	28	1/12	87.5	100
None	EBV 10^{-6}	21	6/12		—
Neuramide (2 U/ml, 12 h)	EBV 10^{-6}	28	1/12	83.3	100
Neuramide (2 U/ml, 24 h)	EBV 10^{-6}	NT	0/12	100	100

U, unit; NT, non-transformed; h, hours after EBV infection, when treatment was started.
[a] Neuramide treatment was started at 12 and 24 h after EBV infection.
[b] Measured after 72 h by cpm.

demonstrates the highly specific blocking activity of neuramide (2 IU/ml) and the increasing effectiveness of the antiviral activity when the time between infection and treatment is increased (compare Tables 1 and 2).

3.3. Effect of neuramide on DNA synthesis and proliferation of EBV-genome-positive cells

To determine whether neuramide could specifically block proliferation of EBV-transformed

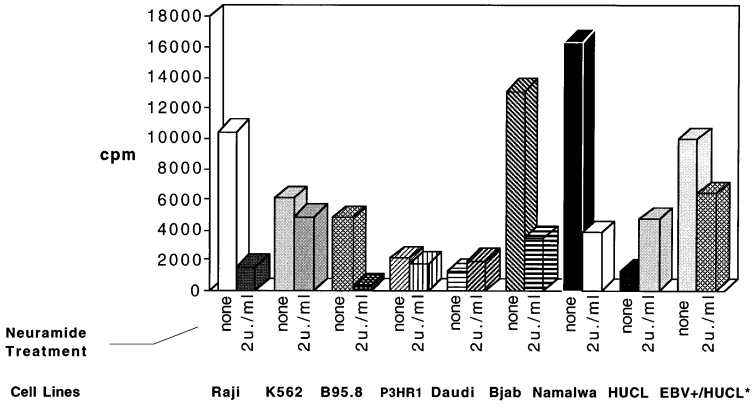

Fig. 1. Inhibiting effect of neuramide on cells that exhibit an EBV-transformed status. Inhibition of EBV-induced DNA synthesis was measured by [^{3}H]thymidine uptake and expressed in cpm. * EBV-immortalized HUCL.

cells, we compared the effect of this drug on DNA synthesis and proliferation in a panel of cells that represent the whole spectrum of EBV–cell interaction. The antiproliferative effect of neuramide was tested in the following cells: (i) normal cells such as uninfected HUCL and HUCL immortalized by the EBV strain B95.8 virus (yet not neoplastic); (ii) EBV-positive cell lines such as Raji, Daudi, P3HR1 and Namalwa (derived from EBV-positive BL); (iii) B95.8 cell line, producer of the EBV transforming strain; and (iv) EBV-negative BL-derived cell lines such as Bjab and BL28, and the EBV counterpart of BL28, E95B-BL28. Finally, to assess neuramide specificity versus EBV-transformed B-cell types, a panel of EBV-negative cell lines such as K562, HuT 78 and Jurkatt, representing erythroblastoid (K562) or T cells (the two others), were used as negative controls (Fig. 1, Table 3).

In Fig. 1, results show that neuramide strongly inhibits proliferation of Namalwa (up to 75.2% inhibition of DNA synthesis), B95.8 and Raji cells, whereas it does not affect growth of P3HR1 and Daudi cells. No effect of neuramide was observed in the EBV-negative cell line K-562. Interestingly, among the EBV-positive cell lines, both Daudi and P3HR1 lack some of the latency proteins. No inhibiting activity was noted against normal HUCL or EBV-immortalized HUCL. A strong inhibiting effect on DNA synthesis was also observed against Bjab cell line (76.3%), which is an EBV-negative lymphoma cell line (Fig. 1).

The same results, with insignificant variations, were obtained with a 6-day treatment with neuramide at the same concentration (data not shown). The resulting antiproliferative effects of neuramide specifically directed against cells exhibiting an EBV-transformed status, have been further confirmed by another set of data (Table 3). We tested the effect of neuramide on the viability of EBV-positive and EBV-negative BL cell lines, and of unrelated T-lymphoid cells used as specificity controls. The percentage increase in mortality over the baseline adding 2 IU/ml of neuramide was measured. In addition, the CC_{50} value, compared with untreated controls, was also determined.

Table 3
Effect of neuramide on the viability of different cell lines

Cell type	EBV genome	% Cell death[a]	CC_{50} (U/ml)[b]
T cell lines			
Jurkat	−	0.0	3.5 ± 0.8
HuT 78	−	5.8	3.0 ± 0.5
Burkitt's lymphoma			
P3HR1	+	61.3	1.7 ± 0.2
Raji	+	33.0	0.2 ± 0.5
BL28	−	8.4	1.8 ± 0.3
E95B-BL28	+	15.7	0.2 ± 0.3

[a] Neuramide concentration: 2 U/ml.
[b] CC_{50} represents 50% cytotoxic concentration, i.e. concentration required to achieve 50% cell death.

EBV-positive BL cell lines show a high death rate in the presence of 2 IU/ml neuramide (33–61.3%), while only a minor (5.8%) or no death rate at all was detected in controls. Interestingly, BL28 (an EBV-negative BL cell line) also showed a death rate about 4–7-fold lower than EBV-positive BLs. However, the death rate almost doubled in the same cell line if EBV-converted (E95B-BL28).

EBV-positive BL cell lines are much more sensitive to neuramide than T cell lines used as controls, since the CC_{50} of the drug for these cells is approximately 2–17-fold lower than for either Jurkat or HuT 78. Furthermore, EBV-negative BL28 cells show a 9-fold higher CC_{50} than for their EBV-converted counterpart E95B-BL28.

4. Discussion

In the present study, we have investigated the antiviral activity of neuramide, and more specifically, its blocking effects on the immortalization process of human B lymphocytes by EBV. We have used a neuramide preparation that is commercially available for human use because it contains all the fractions that have both immunostimulating and antiviral activity. Our results indicate that neuramide specifically blocks EBV-induced growth transformation, but not the early steps of infection, as shown by the differences among pre-, simultaneous and post-treatment. In fact, neuramide shows its greatest inhibiting effect, both on growth transformation and DNA synthesis, when given to cells at 24 h after EBV infection. For HUCL infected by the transforming strain B95.8 of EBV, the simultaneous treatment and post-treatment of neuramide are more effective in inhibiting cellular transformation than pretreatment. Thus, our results suggest that neuramide does not affect the early phases of EBV infection, such as attachment and internalization, but interferes with late mechanisms of transformation that take place between 48 and 72 h postinfection (compare Tables 1 and 2).

All the events which are involved in the transformation of the cells by EBV are not yet fully understood, but they are thought to be initially regulated by viral proteins that are expressed in the early stages of de novo infection. Thus, to inhibit EBV growth transformation activity, neuramide may regulate functions that are affected by early viral proteins, and in such case, post-treatment by the drug would be more effective than pretreatment or simultaneous treatment. Although the exact molecular mechanism of action of neuramide has yet to be determined, we propose that this drug acts against EBV, particularly in the late stages of infection/early stages of transformation when the viral genome is actively expressed. In fact, data on EBV-induced cell growth transformation and DNA synthesis are consistent with the notion that the effect of neuramide is at a maximum between 12 and 24 h after infection, when EBV starts its replication upon the synthesis of the EBNA and determines the fate of the infected cells (Robinson and Smith, 1981; Countryman and Miller, 1985). The effect of neuramide on the late stages of infection is also in accord with previously reported observations that a significant inhibition of influenza A virus by neuramide is obtained up to 5 h postinfection (Antonelli et al., 1986).

The specificity of neuramide is confirmed by the drastic reduction of viability of EBV cell lines versus non-EBV cell lines (Fig. 1, Table 3), and by its minimum antiproliferative effect on non-EBV-infected cell lines such as K562, Jurkat or HuT 78. Apparently, the only exception is represented by the Bjab cell line, derived from an EBV-negative lymphoma: in fact, the drug shows an inhibiting effect (76.3% reduction in DNA synthesis) against Bjab similar to that observed against Namalwa. This latter finding seems to confirm that neuramide's mechanism of action is multipronged and that it may act at several levels of the B-lymphocyte growth transformation pathway, some of which may not be directly related to EBV. It is also likely that different fractions of the drug, which is a mixture of different polypeptides (Miraglia et al., 1990) are responsible for the different effects on B-cell growth transformation mediated by EBV. Our results support the necessity of purification and characterization of the several fractions of neuramide, not only to fully understand its mechanism(s) of action, but also to increase its efficacy, in view of its possible use in vivo in EBV-associated syndromes. For many years, neuramide has been a prescription drug for other herpetic diseases in

Italy. It has no known side effects. Given these in vitro findings, in vivo testing of this drug in the therapy of patients with reactivated EBV-associated diseases may be entertained. It is noteworthy that in the USA, a substance has been recently reported that has many features similar to neuramide. This substance concerns a polypeptide mixture extracted from porcine liver (Ablashi et al., 1994a,b), whose use has recently been proposed against human herpesvirus type 6 (HHV-6), another human lymphotropic herpes virus.

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References

Ablashi, D.V., Berneman, Z.N., Lawyer, C., Kramarsky, B., Ferguson, D.M. and Komaroff, A.L. (1994a) Antiviral activity in vitro of kutapressin against human herpesvirus-6. In-Vivo 8, 581–586.

Ablashi, D.V., Lawyer, C.H., Ferguson, D.M. and Pearson, G.R. (1994b) Antiviral activity of kutapressin against Epstein-Barr virus. In: Epstein-Barr Virus and Associated Diseases, p. 225. Cold Spring Harbor Meeting on Cancer Cells. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Alberini, C.M., De Amici, M., Montagna, D., Guarnaccia, S., Nespoli, L. and Ugazio, A.G. (1982) Immunostimulant effect of neuramide. Potentiation of lymphocyte proliferation and production of immunoglobulins in vitro. J. Ital. Chemioter. 29, 139–144.

Albrecht, T.B., Cole, N., Speelman, D., Dianzani, F. and Baron, S. (1983) Inhibition of varicella-zoster virus replication by neuramide. Chemioterapia 2, 255–256.

Antonelli, G., Dianzani, F., Coppenhaver, D.H. et al. (1986) An influenza virus inhibitor that acts late in the replication cycle. Antimicrob. Agents Chemother. 29, 49–51.

Baron, S. and Albrecht, T. (1982) Protamide—CVI studies—progress report to Cooper Laboratories, March 1982. Department of Microbiology, Medical Branch, University of Texas, TX.

Baron, S. and McKerlie, M.L. (1981) Broadly active inhibitor of viruses spontaneously produced by many cell types in culture. Infect. Immun. 32, 449–453.

Baron, S., Niesel, D., Singh, I.P., McKerlie, L., Poast, J., Chopra, A., Antonelli, G., Dianzani, F. and Coppenhaver, D.H. (1989) Recently described innate broad spectrum virus inhibitors. Microb. Pathog. 7, 237–247.

Bruni, L., Tagliapietra, G. and Innocenti, P. (1984) Herpes zoster treatments: results of a clinical trial relative to the use of rifamycin SV versus neuramide. J. Int. Med. Res. 12, 255–260.

Cirone, M., Angeloni, A., Barile, G., Zompetta, C., Venanzoni, M., Torrisi, M.R., Frati, L. and Faggioni, A. (1990) Epstein-Barr virus internalization and infectivity are blocked by selective protein kinase C inhibitors. Int. J. Cancer 45, 490–493.

Countryman, J. and Miller, G. (1985) Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. Proc. Natl. Acad. Sci. USA 82, 4085–4089.

Garner, J.G., Hirsch, M.S. and Schooley, R.T. (1984) Prevention of Epstein-Barr virus-induced B-cell outgrowth by interferon alpha. Infect. Immun. 43, 920–924.

Lin, J.C., Zhang, Z.X., Chou, T.C., Sim, I. and Pagano, J.S. (1989) Synergistic inhibition of Epstein-Barr virus: transformation of B lymphocytes by α and γ interferon and by 3′-azido-3′-deoxythymidine. J. Infect. Dis. 159, 248–254.

Kumar, S. and Baron, S. (1981) Non-interferon cellular products capable of virus inhibition. Texas Rep. Biol. Med. 41, 395–401.

Kumar, S., McKerlie, M.L., Albrecht, T.B., Goldman, A.S. and Baron, S. (1984) A broadly active viral inhibitor in human and animal organ extracts and body fluids. Proc. Soc. Exp. Biol. Med. 177, 104–111.

Miraglia, N., Rindone, B., Folchitto, G. et al. (1990) High-performance liquid chromatographic approach to the separation of antiviral and immunostimulant fractions in neuramide. J. Chromatogr. 512, 123–129.

Nemerow, G. and Cooper, N. (1984) Infection of B lymphocytes by a human herpesvirus, Epstein-Barr virus, is blocked by calmodulin antagonists. Proc. Natl. Acad. Sci. USA 81, 4955–4959.

Reedman, B. and Klein, G. (1973) Cellular localization of an Epstein-Barr virus (EBV) associated complement-fixing antigen in producer and non-producer lymphoblastoid cell lines. Int. J. Cancer 11, 499–520.

Robinson, J. and Smith, D. (1981) Infection of human B lymphocytes with high multiplicities of Epstein-Barr virus: kinetics of EBNA expression, cellular DNA synthesis, and mitosis. Virology 109, 336–343.

Sixbey, J.W. and Pagano, J.S. (1985) Epstein-Barr virus transformation of human B lymphocytes despite inhibition of viral polymerase. J. Virol. 53, 299–301.