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Inhibitory and Cytotoxic Effects of Oncovin (Vincristine Sulfate) on Cells of Human Line NHIK 3025[1]

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SUMMARY

The inhibitory effect of Oncovin (vincristine sulfate) on cell division was studied in human cell line NHIK 3025. Oncovin arrested the cells in metaphase at concentrations as low as 10^{-3} μg/ml. At 8×10^{-3} μg/ml and higher concentrations, the arrest was complete after 6 hr of treatment. The arrest was irreversible after exposure for 6 hr to 16×10^{-3} μg Oncovin per ml.

The X-ray radiosensitivity of aerobic cells of the same line pretreated with 16×10^{-3} μg Oncovin per ml for 6 hr (Oncovin removed before irradiation) was found to be about equal to that of untreated cells.

Even when present during irradiation, Oncovin did not exert any modifying effect on the radiosensitivity of either aerobic or extremely hypoxic cells.

INTRODUCTION

Oncovin (vincristine sulfate) is an alkaloid from the periwinkle (*Vinca rosea* Linn.) (8). The substance is known as a mitotic inhibitor that, in several cell types, both *in vivo* and *in vitro* (5, 6), arrests the cell cycle in metaphase. The exact mechanism of this stathmokinetic effect is still unknown. During recent years it has been established that Oncovin prevents the formation of the spindle by precipitating the microtubular protein (1, 11). Other reports suggest that this disruption of the cell division may be caused by inhibition of the RNA, protein, and DNA synthesis (2, 3).

Oncovin has been shown to produce a marked regression in a wide spectrum of human tumors (7). Today it is used mostly in combination with other chemotherapeutic agents (4). Some attempts have been made to obtain better results by combining Oncovin treatment with irradiation (13, 22, 23). Oncovin has little effect on the bone marrow and may, from this point of view, be suitable in combination therapy.

The 1st part of this report deals with the inhibitory effect of Oncovin in different concentrations on cell division of a human cell line (NHIK 3025) cultivated *in vitro*. The 2nd part deals with the effect upon the radiosensitivity of these cells, with the drug present during irradiation under aerobic and extremely hypoxic conditions (<4 ppm O_2), and with the drug removed prior to irradiation under aerobic conditions.

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MATERIALS AND METHODS

One mg of Oncovin (Eli Lilly and Company, Indianapolis, Ind.), stored in ampuls, was dissolved in 100 ml Hanks' solution (20). The desired concentrations were obtained by further dilutions in Tissue Culture Medium E2a (20).

Human cell line NHIK 3025, derived from an early stage of cervical carcinoma, was used (14, 16). The doubling time of these cells is about 22 hr.

The method used for determination of the effect of Oncovin on cell division has previously been described (15). At the start of an experiment, cells from stock cultures were suspended in Medium E2a in a concentration of 200,000 cells/ml after trypsinization. One ml of the suspension was added to each tube, in which a strip of Corning coverglass had been fastened. The tubes were placed horizontally at 37° for 24 hr. The medium was then replaced with 1 ml fresh medium, and the tubes were again placed in a horizontal position. The next day the medium was replaced with 1 ml fresh medium containing Oncovin in the concentrations to be tested. The cells were fixed in Carnoy's fluid and stained according to Bøhmer's method with hematoxylin (21). One thousand cells/slide were examined microscopically, and the numbers of cells in different stages (prophase, metaphase, anaphase, telophase, and interphase) were noted. Only whole cells were scored. Each point in the diagrams (Charts 2 and 3) represents the mean of observations based on 5 slides, except for a few cases, in which the number was 3 or 4. Standard errors were calculated for all groups and can be seen in the illustrations, except when the symbols are smaller than those used for the mean values.

In Charts 4 and 5, each experimental point represents the mean value of the survival ratios, based on from 1 to 4 completely independent sets of experiments.

In irradiation experiments, surviving fractions were measured by clonogenic assays. The techniques employed are described elsewhere (18, 19). A sketch of the degassing setup for preparation of extremely hypoxic cells is shown in Chart 1. The setup is made of glass and stainless steel. The gas mixture of 97% N_2 and 3% CO_2 (containing less than 4 ppm O_2) was humidified in a water bath before it entered the irradiation chamber. Six Petri dishes were placed in the irradiation chamber. The cells were attached to the bottom of the dishes and covered with only 1 ml growth medium (0.4-mm depth). In 3 of the dishes, the medium contained Oncovin; the other 3 were controls. The effluent gas from the irradiation chamber passed through an open water lock to prevent oxygen from diffusing into the irradiation cham-

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ber through the steel pipe. Five % of the outflowing gas could be led through a Hersch oxygen meter calibrated for oxygen concentrations between 0 and 10 ppm. The high-purity nitrogen and CO_2 gas used in these experiments (obtained from Norsk Hydro A/S, Oslo, Norway) had a maximum concentration of oxygen of 3 ppm. Degassing time before irradiation was 30 min, the volume of the irradiation chambers was 2.4 liters, and the flow was 5 liters/min.

The radiomodifying effect of Oncovin was tested under 5 different conditions. (*a*) Cells pretreated with Oncovin for 6 hr were irradiated in the absence of Oncovin under aerobic conditions either in suspension or attached to glass dishes. A detailed description of these 2 techniques has been given previously (18). (*b*) Cells pretreated with Oncovin for 6 hr were irradiated in the presence of Oncovin under aerobic conditions in suspension. (*c*) Cells not pretreated with Oncovin were irradiated either in the absence or in the presence of Oncovin under extremely hypoxic conditions, attached to glass dishes.

Cultures of cells taken for experiments were always 3 days old (in log phase). Irradiation was performed by a Siemens Stabilipan X-ray unit operated at 220 kV, 20 ma, and with 0.5-mm Cu half-value layer filtration.

The dose rate was determined by Fricke dosimeter solution (12) to be 300 rads/min for aerobic cells irradiated in suspension and 363 rads/min for aerobic cells irradiated attached to glass dishes. For extremely hypoxic cells, the dose rate was about 215 rads/min.

The reason that the dose rate was higher for cells irradiated under aerobic conditions than for cells irradiated under extremely hypoxic conditions was that the cells were not kept inside the steel chamber under the aerobic irradiations; the radiation was therefore not filtered by the steel lid of the chamber.

RESULTS AND DISCUSSION

Inhibition of Cell Proliferation. Chart 2 shows the effect of 6 hr of treatment with Oncovin in 14 different concentrations ranging from 0.03×10^{-3} to 0.5 μg/ml. There was no change in the metaphase index (number of metaphases per 1000 cells) at concentrations lower than 0.5×10^{-3} μg/ml (*Curve A*). At 10^{-3} μg/ml, a significant arrest of cells in metaphase was observed. The metaphase index increased with increasing concentrations up to about 8×10^{-3} μg Oncovin per ml. At this and higher concentrations, the arrest was complete. No anaphase or telophase could be seen (*Curve B*). For concentrations exceeding 32×10^{-3}, the metaphase index was slightly reduced, compared with the maximal value. At all concentrations, prophases were found to the same extent as in the control (*Curve C*).

Chart 3 shows the effect on metaphase index of Oncovin at concentrations 10^{-3}, 2×10^{-3}, 16×10^{-3}, and 0.128 μg/ml over a period of up to 24 hr. The metaphase index of the control group was constant during this time, as expected for cells in log phase.

At 10^{-3} μg Oncovin per ml, the metaphase index reached a maximum value of about 100 after 9 hr. At this concentration, the arrest was not complete, since anaphases and telophases were found.

At 2×10^{-3} μg/ml, the metaphase index reached a maximum value of slightly less than 200 after 9 hr. After 24 hr, the metaphase index had decreased to about 135, and some anaphases and telophases were found. At this time, several multinucleated cells were observed. Many cells had obviously completed mitosis, some in an irregular way.

At 16×10^{-3} μg/ml, an enormous increase of the metaphase index after 24 hr was observed, with about 60% of the

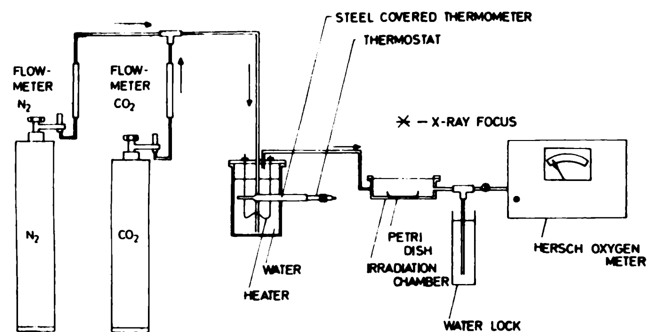

Chart 1. Setup for preparation of extremely hypoxic cells (see Text).

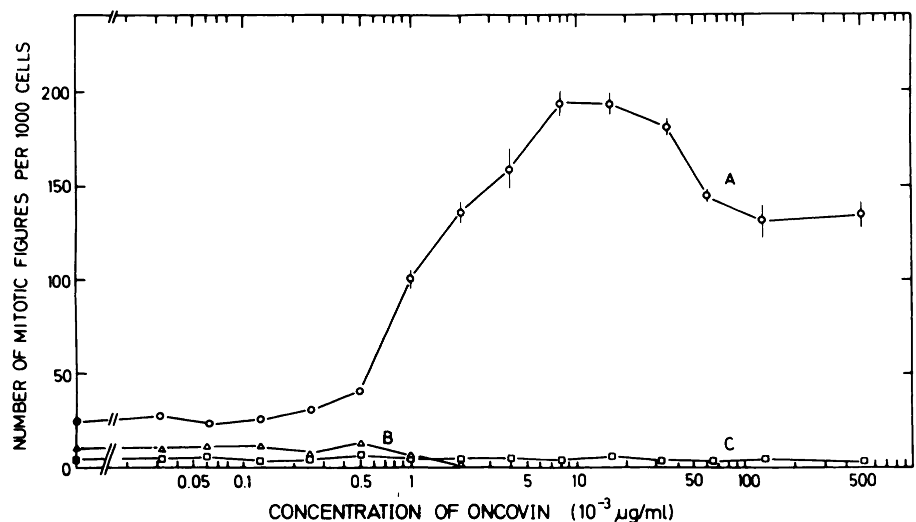

Chart 2. The prophase (*C*), metaphase (*A*), and anaphase + telophase (*B*) indices, with standard errors, are shown after 6 hr of treatment of NHIK 3025 cells with Oncovin in different concentrations.

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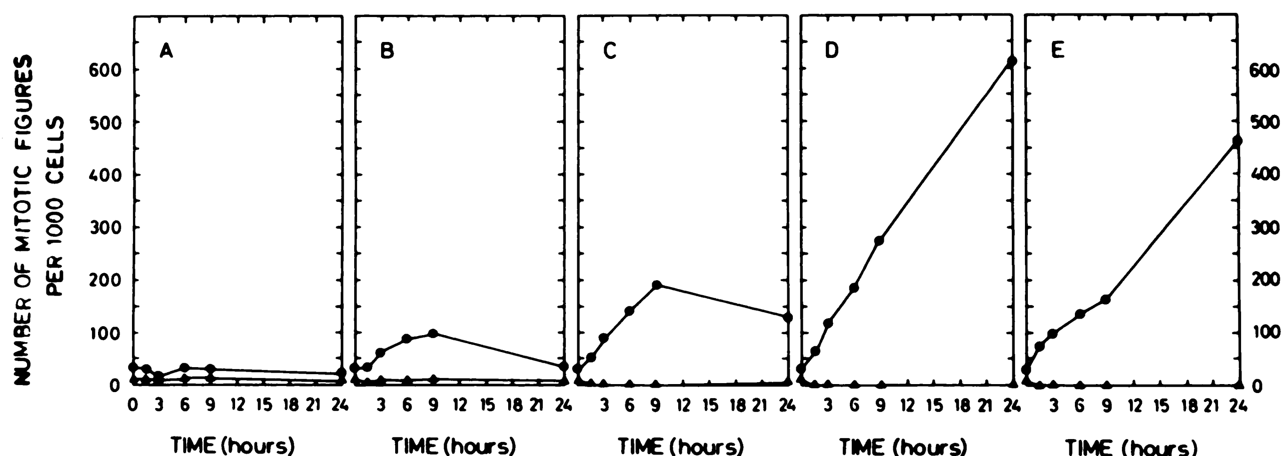

Chart 3. The metaphase index (●), and anaphase + telophase index (▲), with standard errors, after treatment for times up to 24 hr with Oncovin of the following concentrations: *A*, control; *B*, 10^{-3} µg Oncovin per ml; *C*, 2×10^{-3} µg Oncovin per ml; *D*, 16×10^{-3} µg Oncovin per ml; *E*, 0.128 µg Oncovin per ml.

cells arrested in metaphase. At this concentration, the arrest was complete and thus led to an accumulation of all metaphases with time. After 24 hr, some destruction of metaphases could be seen, and the cells in interphase were more granulated than normal cells.

At 0.128 µg/ml, the metaphase index increased with time during the 24-hr period. Close to 50% of the cells were arrested in metaphase after 24 hr. After only 1.5 hr, no anaphase and telophase were found. After 24 hr, severe destruction of metaphase was observed, and a reduced number of cells as compared with that of the control was attached to these slides, indicating a selective detachment of metaphases. The individual counts showed also a higher degree of variation compared with the other groups. Thus, the true value of the metaphase index is probably higher than that scored.

In most of the arrested metaphases, the chromosomes were assembled in the middle of the cell (ball metaphase). In others, the chromosomes were irregularly scattered in the cytoplasm. High concentrations and long exposures to the drug lead to pyknosis.

The reduction of the metaphase index at concentrations higher than 32×10^{-3} µg/ml (Chart 2), as compared with the optimal accumulation, may have been caused by (*a*) destruction of metaphases or (*b*) inhibitory effect in interphase preventing some cells from entering mitosis.

Destruction of metaphases was observed with the highest concentrations after 6 hr of treatment. It is, however, impossible from this experiment to determine whether there really is an inhibition in interphase for high concentrations of the drug. Other experiments (Chart 3) showed that about 50% of the cells exposed to 0.128 µg Oncovin per ml were arrested in metaphase. Considering the doubling time of the cells, this relatively low value of the metaphase index after 24 hr might indicate that some cells are prevented from entering mitosis or possibly a progression delay.

Madoc-Jones and Mauro (10) have studied the interphase action of Oncovin on HeLa and Chinese hamster cells. They found that Oncovin exerted a differential lethal action depending upon the position of the cells in interphase at concentrations higher than those needed for mitotic arrest. The cells in S phase were more sensitive than cells in other positions of interphase.

Johnson *et al.* (9) have published results that might indicate an inhibition in interphase for higher concentrations of the drug. In cultures of Chinese hamster ovarian cells, more mitotic figures were observed with 0.02 µg/ml (318 metaphases/100 metaphases in the control) than with 0.2 µg/ml (244 metaphases/100 metaphases in the control).

George *et al.* (6) have studied the effect of Oncovin on mitosis in HeLa cells at concentrations from 10^{-8} to 1 µg/ml. They found that, at 10^{-3} µg/ml, most of the dividing cells were arrested in metaphase. The same concentrations were found to produce an arrest in metaphase in NHIK 3025 cells. Exposure of HeLa cells to 1 and 0.1 µg of Oncovin per ml from 0.5 to 20 hr resulted in an arrest that could not be reversed by repeated washings of the cultures with fresh medium. By electron microscopy they observed that the spindle tubules were absent in the arrested cells.

Reversibility of Proliferation Inhibition. An experiment was carried out to test whether the arrest in metaphase was reversible after treatment with Oncovin, 16×10^{-3} µg/ml, for 6 hr. Cells grown for 6 hr in the presence of Oncovin at this concentration were incubated with fresh medium. To assure better removal of Oncovin, the medium was changed once more after 5 min. The metaphase index was determined 20, 40, 60, and 90 min after removal of the inhibitor. The metaphase index increased during the period of 90 min from about 200 to nearly 240. Hence, the arrest in metaphase of cells exposed to 16×10^{-3} µg Oncovin per ml for 6 hr seems to be irreverisble.

Combined Effects of Oncovin and X-rays. To establish a quantitative relationship between cellular metaphase arrest and cellular viability, experiments comparing the radiosensitivity of a cell population of untreated cells with that of a population treated with Oncovin were performed. NHIK 3025 cells exposed to 16×10^{-3} µg/ml for 6 hr had about a 50% reduction in plating efficiency compared with the control cells (no Oncovin).

To determine the radiosensitivity under aerobic conditions of NHIK 3025 cells surviving Oncovin treatment, the 2 types of experiments described under *a* and *b* in "Materials and Methods" were performed with a concentration of Oncovin of 16×10^{-3} µg/ml.

Chart 4 shows the results of experiments in which cells under aerobic conditions and pretreated with Oncovin, 16 ×

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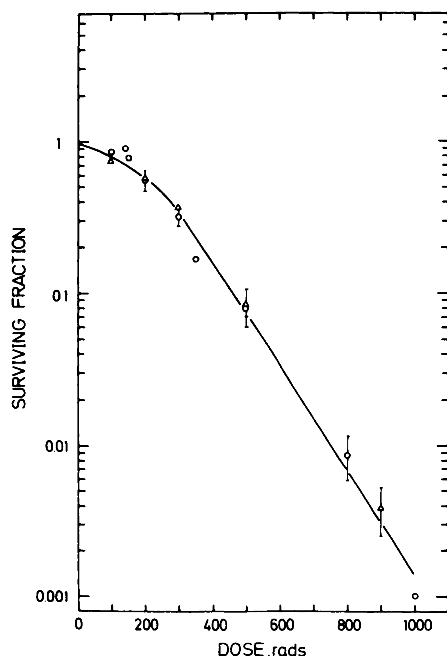

Chart 4. Survival data for aerobic NHIK 3025 cells pretreated with 16 × 10^{-3} µg Oncovin per ml for 6 hr and irradiated in absence of Oncovin. O, cells irradiated attached to glass dishes; Δ, cells irradiated in suspension. The curve drawn is that determined by Pettersen *et al.* (19) for untreated cell population and with the same technique as here used for cells fastened to glass.

10^{-3} µg/ml, for 6 hr were irradiated under 2 experimental conditions: (*a*) fastened to glass surface and (*b*) in suspension. In none of these experiments was Oncovin present during irradiation. The curve drawn is the survival curve determined for untreated cells under otherwise identical conditions to those in *a* (19). The exponential part has been fitted by the method of least squares.

Both survival curves have a shoulder for low doses followed by an exponential part for doses from 300 to 900 rads. The survival curve of untreated cells has an extrapolation number of 3.8 and D_0 = 130 (found by the method of least squares). The survival curve of pretreated cells irradiated when fastened to a glass surface has an extrapolation number of 3.4 and D_0 = 135, while those irradiated in suspension have an extrapolation number of 4.4 and D_0 = 130. These results indicate that there is no significant difference between the radiosensitivity of untreated cells and that of pretreated cells under aerobic conditions.

Chart 5 shows the results of experiments in which cells were pretreated with, and irradiated in the presence of, 16 × 10^{-3} µg Oncovin per ml. The curve drawn is the survival curve for untreated cells under otherwise identical conditions (18). For comparison, the data for pretreated cells irradiated in suspension (medium) are presented in the same chart. The data indicate that Oncovin does not affect the radiosensitivity of the cells when present during irradiation.

Terasima and Tolmach (24) have published age-response curves after irradiation of synchronized aerobic HeLa cells. They found that the cells were most resistant in early and middle G_1 phase and late S and G_2 phase. Similar experiments recently performed with synchronized cultures of NHIK 3025 cells showed the cells to be resistant in middle G_1 and in late S. The G_1 phase lasts for about 7 to 9 hr.

Although cells pretreated with Oncovin, 16 × 10^{-3} µg/ml, for 6 hr are partially synchronized, we did not find any significant difference in the radiosensitivity of pretreated and untreated NHIK 3025 cells. The reason for this might be that radiosensitive cells in mitosis and early G_1, as well as radioresistant cells in mid-G_1 (17), are inactivated by Oncovin in such a way that the radiation response of our pretreated population is little different from that of an untreated population.

Since parts of certain tumors consist of radioresistant hypoxic cells, it is of interest to investigate whether Oncovin

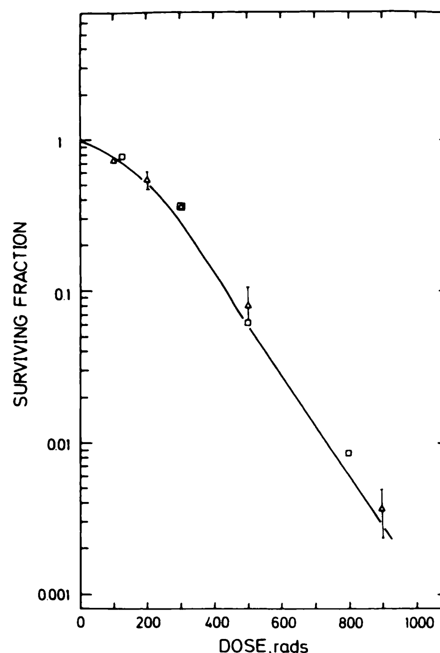

Chart 5. Survival data for aerobic NHIK 3025 cells pretreated with 16 × 10^{-3} µg Oncovin per ml for 6 hr and irradiated in suspension in presence of Oncovin of the same concentration. Δ, cells irradiated in medium without Oncovin; □, cells irradiated in medium containing 16 × 10^{-3} µg Oncovin per ml. The curve drawn has been determined by Pettersen *et al.* (18) for an untreated cell population irradiated in suspension.

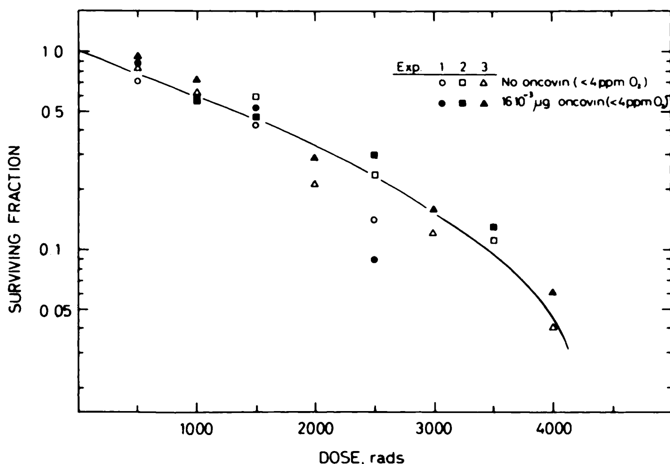

Chart 6. Survival data for extremely hypoxic NHIK 3025 cells irradiated attached to glass dishes in absence and in presence of 16 × 10^{-3} µg Oncovin per ml. The data represent 3 independent experiments (*Exp.*). Oncovin was added to the cells about 40 min before irradiation and was removed before the cells were plated.

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has a modifying effect on the radiosensitivity of such cells. A radiosensitizing effect by the drug would be desirable in combination therapy with Oncovin and radiation. Chart 6 shows data from 3 experiments in which extremely hypoxic cells (<4 ppm O_2) have been irradiated in the presence and in the absence of Oncovin at a concentration of 16×10^{-3} μg/ml. Dishes irradiated with and without Oncovin were degassed and irradiated simultaneously in the same irradiation chamber.

Chart 6 shows no significant effect of Oncovin upon the dose-survival ratios at any dose level studied. Thus, the present data suggest that it is unlikely that Oncovin acts as a radiosensitizing agent of hypoxic cells in radiotherapy of cervical carcinoma.

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