Age-dependent Cell Inactivation by Vincristine Alone or in Combination with 1-Propargyl-5-chloropyrimidin-2-one

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ABSTRACT

Inactivating effects caused by vincristine alone or in combination with another mitotic inhibitor, 1-propargyl-5-chloropyrimidin-2-one, were studied as loss of colony-forming ability in exponentially growing or synchronized populations of the human cell line NHIK 3025.

Treatment with 4 ng vincristine per ml (4.3 nm) in G2 led to irreversible mitotic arrest. Both mitotic arrest and lethal damage due to vincristine were primarily induced when cells were exposed in late S and G2, suggesting a correlation between the cell cycle-inhibitory and inactivating effects of this drug at clinically relevant concentrations. No repair of sublethal damage after vincristine treatment could be detected within 5 hr.

A common feature in the age response of NHIK 3025 cells to the two mitotic inhibitors is drug resistance in G1. However, while mitosis is the most sensitive stage in the cycle with respect to inactivation by 1-propargyl-5-chloropyrimidin-2-one, mitotic cells are relatively resistant to treatment with vincristine. The combined inactivating effect of vincristine and 1-propargyl-5-chloropyrimidin-2-one was purely additive during interphase. In mitosis, the two drugs demonstrated a striking synergistic effect.

INTRODUCTION

Although the metaphase arrest caused by treatment with different mitotic inhibitors is generally reversible, several investigators have shown that treatment with the Vinca alkaloid vincristine entails irreversible mitotic arrest (4). Madoc-Jones and Mauro (10) demonstrated that HeLa cells exposed to a high concentration of vincristine (100 ng/ml) in S phase proceeded at a normal rate to mitosis, where they were arrested irreversibly. However, these investigators also reported that mitotic cells were relatively resistant to a 3-hr exposure to vincristine (12). Studies of a number of different cell populations both in vitro (5) and in vivo (3, 8) have demonstrated an immediate rise (within 1 to 2 hr after addition) in the mitotic index after treatment with vincristine, suggesting that mitotic arrest is not only induced in S phase but also in G2. This leads to the first question I wanted to answer during this investigation: 1. To what extent is mitotic arrest following treatment with clinically attainable concentrations of vincristine irreversible? 2. Where in the cell cycle is mitotic arrest caused by vincristine induced?

For many chemotherapeutic drugs, there seems to be no correlation between lethal cell damage and cell cycle-inhibitory effects (1). In his review article from 1972, Marsden (11) stated that the mechanism behind the oncolytic effect of vincristine was not completely understood, and this is still the situation today. Rosner et al. (17) postulated that, at low concentrations, vincristine cytotoxicity is not the result of metaphase arrest. This suggestion was supported by Olah et al. (14), showing that Chinese hamster cells were more sensitive to vincristine in plateau than in log phase, indicating that cell inactivation by vincristine is not dependent on the traverse of cells through the division cycle. Consequently, the next question to be answered in this report is: 3. Is there a connection between mitotic arrest and lethal effects caused by clinically relevant concentrations of vincristine?

Earlier reports from our laboratory (20, 21) have shown that the metaphase-arresting properties of the mitotic inhibitor NY 31702 are similar to those of vincristine, while inhibitory effects in interphase seem to be rather different for these 2 drugs. Age-dependent cell inactivation by NY 3170 has also been demonstrated (19). The last questions are therefore: 4. Is there any similarity between the lethal actions of the 2 mitotic inhibitors vincristine and NY 3170? 5. Do these 2 drugs demonstrate a synergistic or antagonistic effect anywhere in the cell cycle?

MATERIALS AND METHODS

Mitotic Inhibitors. Vincristine (Oncovin) was purchased from Eli Lilly and Co., Indianapolis, Ind. Stock solutions (10 μg vincristine per ml in Hanks' solution; 1 ng/ml = 1.08 nm) were made weekly and stored at 4°C.

The chemical structure of NY 3170 (M.W. 169) has been presented in a previous paper (21). This drug was synthesized at the University of Oslo (9). NY 3170 was dissolved in medium before each experiment, and the drug solution was sterilized by Millipore filtration.

Cell Culture. The cell line used in this study, NHIK 3025, originates from a human cervical carcinoma in situ. The cells were grown in Medium E2a (16) supplemented with 20% human and 10% horse serum. Under these conditions, the average generation time of NHIK 3025 cells is 18 hr, which is also the population-doubling time (15). The average durations of G1, S, G2, and mitosis of synchronized cells are 6.5, 8, 2.5, and 1 hr, respectively (15).

Cell inactivation was measured as loss of colony-forming ability. Colonies containing more than 40 cells after the required time of incubation (10 to 12 days) were scored as viable. The medium was changed 6 to 7 days after plating. The colonies were fixed (absolute ethanol) and stained (methylene blue) before counting.

1 The abbreviation used is: NY 3170, 1-propargyl-5-chloropyrimidin-2-one.

2 The abbreviation used is: NY 3170, 1-propargyl-5-chloropyrimidin-2-one.

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In each of the charts, the results from one typical experiment are shown. Standard errors of colony numbers per dish or flask in each experimental group are indicated when they exceed the size of the symbols. All experiments were repeated 1 to 4 times, yielding practically the same result.

**Dose-Response Studies.** Cells from an exponentially growing population were plated in Petri dishes (5 replicate dishes in each experimental group; 200 to 1000 cells plated per dish). Due to frequent reculture of stock cultures (trypsinized 3 times weekly), the initial lag after plating was only about 1 hr. Different concentrations of vincristine were added by change of medium after an attachment period of 2 hr. One hr later, the medium containing vincristine was removed, the dishes were rinsed 3 times with Hanks’ solution, and fresh medium was added.

**Age-Response Studies.** Synchronized cell populations were obtained by the method of mitotic selection (15). The newly selected cells were allowed to attach to the bottom of 25-cm tissue culture flasks (2 replicate flasks; 250 cells/flask). At set times after mitotic selection, the cells were exposed for 1 hr to fresh control medium, vincristine, NY 3170, or both drugs in combination, by change of medium. Then the flasks were rinsed 3 times with Hanks’ solution, supplied with fresh medium, and incubated for test of viability. To obtain single-cell surviving fractions, corrections for a cell multiplicity of 2 were made as described in a previous paper (19).

At the same time as cells were plated for test of single-cell survival, newly selected cells were also seeded in 25-cm tissue culture flasks for mitotic index measurements. A discrete 3-hr exposure to 4 ng vincristine per ml was performed in different stages of the cell cycle. When the cells reached mitosis, mitotic index (100 x number of mitotic cells/total number of counted cells) was recorded at regular intervals in each of these populations by counting in an inverted microscope with phase-contrast equipment. At least 300 cells were scored for each mitotic index recording.

**Test for Reversibility of Metaphase Arrest.** A new method of measuring reversibility of metaphase arrest was introduced in a previous paper (19). Briefly, an exponentially growing cell population is exposed to a mitotic inhibitor for a set time, after which cells newly arrested in metaphase are selected by shake-off. Two preliminary shake-offs performed 0.5 hr before exposure prevent contamination of the solution of selected cells by loose dead cells or by cells having been arrested in mitosis for more than 0.5 hr.

In some experiments (Chart 3), 4 ng vincristine per ml were added to a flask containing exponentially growing cells by change of medium 3 hr before the final shake-off. Every 0.5 hr later, 4 ng vincristine per ml were added to an identical flask with exponentially growing cells. One flask with untreated cells served as a control. Immediately after shake-off, vincristine was removed by centrifugation, and the cells were plated in Petri dishes (5 replicate dishes) previously filled with fresh medium for test of viability. The number of cells plated was determined by hemacytometer counting for each experimental group.

In another series of experiments (Chart 4), cells were treated with mitotic inhibitors both in G2 (from 3 hr before shake-off) and for various times after entry into mitosis. After shake-off, the cells were kept in suspension in the presence of mitotic inhibitors until plating. When introduced after shake-off, vincristine was added as a 10 x solution in medium. Before plating in Petri dishes (5 replicate dishes), the drugs were removed by centrifugation.

**RESULTS**

Chart 1 presents a survival curve of exponentially growing cells exposed to different concentrations of vincristine for 1 hr. This curve was adapted by a computer program by the method of least squares. The nearly biphasic shape of the survival curve indicates cell cycle phase-dependent inactivation (7). Extrapolation of the latter part of the curve leads to intersection with the ordinate at about 0.35, indicating a resistant fraction counting about 35% of the cells in the exponentially growing population (later shown to be mitotic and G1 cells). In all the experiments of this type, a small fraction (about 0.001) survived exposure to 256 ng/ml (277 nM) for 1 hr, suggesting the presence of a number of extremely resistant cells (evidently cells in the middle of G1; see Chart 2).

The age-response curve with respect to lethal effects caused by vincristine (Chart 2B) confirms the suggestion of a striking cell cycle phase-dependent inactivating effect of this drug. Only G1 cells were resistant to this concentration of vincristine (16 ng/ml; 17.3 nM), while late S and G2 cells appeared to be very sensitive. Mitotic cells were rather resistant, although relatively poor synchrony in the last part of the cycle might conceal a possible “fine structure” in the age-response curve.

Chart 2A shows the maximum mitotic index values measured in the first mitosis after the exposure of synchronized cell populations to 4 ng vincristine per ml (4.3 nM) for 3 hr in different stages of interphase. At this low concentration of vincristine, the progression rate of NHIK 3025 cells through interphase is unaffected (20). Consequently, all the treated
Chart 2. A, maximum mitotic index in populations of NHIK 3025 cells measured during the first mitosis after treatment with 4 ng vincristine per ml (4.3 nM) for 3 hr in different stages of the cell cycle. The measured value for each population is plotted in the middle of the exposure period. The last plotted value shows the maximum mitotic index in a population where 4 ng vincristine per ml were added at 18 hr and not removed. B, single-cell surviving fractions in populations of NHIK 3025 cells measured as colony-forming ability after treatment with 16 ng vincristine per ml (17.3 nM) for 1 hr in different stages of the cell cycle. The measured values are plotted at the times at which exposure began. S.E. is indicated when it exceeds the size of the symbol. Both curves are from the same experiment.

populations reached mitosis simultaneously with the control population. However, cells treated with vincristine in interphase were delayed in mitosis. Accumulation of mitotic cells led to a rise in the mitotic index, and the mitotic index curves did not decline until the number of cells escaping the mitotic arrest was greater than the number of cells entering mitosis. A long average duration of the mitotic arrest was, therefore, reflected in a high maximum mitotic index value. In this way, the degree of mitotic inhibition induced in a cell population could be measured by recording the maximum mitotic index value.

From Chart 2A, it can be seen that cells exposed to 4 ng vincristine per ml in G1 (3 to 6 hr after mitotic selection) were only slightly delayed in mitosis (the maximum mitotic index in the control population was 11%). The induced mitotic inhibition was gradually enhanced as the exposure took place later in the cell cycle. Maximum inhibition appeared after exposure to vincristine in late S-G2 (15 to 18 hr).

Chart 2B demonstrates that G2 cells are sensitive, while mitotic cells are rather resistant with respect to inactivating effects of vincristine. To check whether the resistance to vincristine inactivation depended on the cells having passed one specific stage somewhere in G2, the survival of cell populations exposed to 4 ng vincristine per/ml for different intervals in G2 was measured (Chart 3). The change in sensitivity could not be traced to one specific stage. The survival decreased gradually as the exposure time was increased from 0.5 hr (70% survival) to 3 hr (1% survival).

Chart 4 shows data on the reversibility of the mitotic arrest as a function of the duration of the arrest. Cells were plated for colony formation immediately after having entered mitosis or were kept in metaphase arrest by the presence of mitotic inhibitors for 1, 2, or 4 hr before plating. Chart 4 (○) demonstrates that cells treated with 4 ng vincristine per ml in G2 suffered additional lethal damage when they were kept in mitotic arrest up to 4 hr. Chart 4 (□) shows that 4 ng vincristine per ml had very little effect on cell survival when added after the entry of the cells into metaphase, confirming the impression that mitotic cells are resistant to vincristine with respect to lethal damage.

Chart 4 (dashed curve) shows the survival of NHIK 3025 cells exposed to 0.2 mM NY 3170 alone from 3 hr before mitosis and for various times after entry into metaphase arrest. This curve is redrawn from a previous report (19). Chart 4 (△) shows surviving fractions of cells treated with 0.2 mM NY 3170 from 3 hr before mitosis, and additionally with 4 ng vincristine per ml from shortly after having entered metaphase. The 3-hr G2 exposure to NY 3170 per se had virtually no influence on cell viability, but the combined effect of NY 3170 and vincristine in mitosis led to a rapid decrease in survival with time.

Chart 5 presents data which demonstrate the combined lethal effect of vincristine and NY 3170 during the cell cycle. Chart 5 (dashed curve) represents a theoretical age-response curve indicating perfect additive effect of vincristine and NY 3170 throughout the cycle, when an additive response is defined as described by Drewinko et al. (6). At each time point, the geometric sum of the surviving fractions following 1-hr treatment with 16 ng vincristine per ml and 1-hr treatment with 2 mM NY 3170 was calculated, and the dashed curve was
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vincristine than were both exponentially growing and plateau-phase Chinese hamster cells. A previous report from our laboratory (20) has shown that continuous exposure to 16 ng vincristine per ml (17.3 nM) results in complete metaphase block in NHIK 3025 cells and that no colony formation occurs after continuous exposure to vincristine concentrations above 2 ng per ml (2.2 nM).

The data presented in Chart 2 demonstrate a close correlation between inhibitory and inactivating effects of vincristine with respect to the specific stages in the cell cycle where these effects are induced. Induction of both mitotic arrest and lethal damage takes place primarily in late S-G2. In addition, an earlier report (20) shows that vincristine treatment (64 ng/ml; 69 nM) entails a G2-specific interphase prolongation in NHIK 3025 cells. Consequently, late S-G2 is by far the most sensitive stage in the cell cycle of NHIK 3025 cells with respect to inactivation as well as to inhibition of cycle progression, which suggests that inhibitory and inactivating effects of vincristine may be based on the same mechanism in proliferating cells. Correlation between mitotic spindle dissolution and inactivating effect has been demonstrated for the related drug vinblastine (18). Previous reports from our laboratory (19, 20) have shown that mitotic arrest caused by NY 3170 is induced if, and only if, this drug is present during mitosis and that mitosis is also the most sensitive stage with respect to inactivation by NY 3170. This also indicates a correlation between mitotic inhibition and inactivating effect for NY 3170.

The induction of protracted mitotic arrest after exposure to 4 ng vincristine per ml in G2 (Chart 2A) explains the immediate rise in the mitotic index observed in vitro and in vivo by other

![Chart 4](image)

**Chart 4.** Surviving fractions after treatment of NHIK 3025 cells with mitotic inhibitors in G2 and for various times after entry into mitosis. Bars, S.E. □, cells treated with 4 ng vincristine per ml (4.3 nM) from 3 hr before mitosis. Vincristine was removed immediately after entry into mitosis, or 1, 2, or 4 hr later. ○, cells treated with 4 ng vincristine per ml after having entered mitosis and removed 1, 2, or 4 hr later (untreated in G2). △, cells treated with 0.2 mM NY 3170 from 3 hr before mitosis, and additionally with 4 ng vincristine per ml after having entered mitosis. The drugs were removed 1, 2, or 4 hr after addition of vincristine. ––––, cells treated with 0.2 mM NY 3170 from 3 hr before mitosis. NY 3170 was removed at various times after the cells had entered mitosis. This curve is redrawn from a previous report (19).

drawn between these theoretical survival values. This curve would be the result of the combination of 16 ng vincristine per ml and 2 mM NY 3170, if these 2 drugs worked quite independently with respect to lethal effects. One can see from Chart 5 that the actually measured surviving fractions resulting from treatment with 16 ng vincristine per ml in combination with 2 mM NY 3170 are very close to the calculated values, representing a pure additive effect.

In the experiments performed in this study, cells surviving treatment generally produced colonies of normal control size. This indicates that the treated cells had managed to recover completely. Split-dose experiments with vincristine were performed (data not shown here) to see if there might be any repair of sublethal damage after treatment with this drug. No such recovery could be detected during a 5-hr interval.

**DISCUSSION**

The survival curve of NHIK 3025 cells exposed to vincristine (Chart 1) has approximately the same shape as the one reported by Olah et al. (14) for Chinese hamster cells exposed to this drug. However, NHIK 3025 cells were more sensitive to vincristine than were both exponentially growing and plateau-phase Chinese hamster cells. A previous report from our laboratory (20) has shown that continuous exposure to 16 ng vincristine per ml (17.3 nM) results in complete metaphase block in NHIK 3025 cells and that no colony formation occurs after continuous exposure to vincristine concentrations above 2 ng per ml (2.2 nM).

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![Chart 5](image)

**Chart 5.** Age-response curves of NHIK 3025 cells after 1 hr treatment with 16 ng vincristine per ml (17.3 nM) (A), 2 mM NY 3170 (B), or the combination of 16 ng vincristine per ml and 2 mM NY 3170 (C). Surviving fractions are plotted at the times after mitotic selection at which exposures began. Survival after the exposure to the drug combination, which commenced at 16 hr, was too low to be measured. S.E.'s are indicated when they exceed the size of the symbols. ––––, theoretical age-response curve which would be the result of the drug combination if the effects of vincristine and NY 3170 were purely additive (see "Results").
investigators (3, 5, 8) when asynchronously growing populations were exposed to vincristine.

The age-response curves presented by Madoc-Jones and Mauro (10) of HeLa and Chinese hamster cells exposed to vincristine are quite similar to the age-response curve of NHIK 3025 cells (Chart 2B). However, these investigators concluded that S was the only sensitive stage in the cell cycle with respect to lethal damage. For HeLa cells they showed explicitly (by dose-response curves for synchronized populations) that G1 and G2 cells were equally resistant to vincristine over a wide dose range.

The results presented in Charts 3 and 4 leave little doubt that NHIK 3025 cells in G2 are extremely sensitive to vincristine. This G2 sensitivity indicates that irreversible mitotic arrest from vincristine treatment is not related to DNA synthesis. Inactivation by vincristine seems to be due to interference with events normally taking place in the last part of the cycle but which are completed before the onset of metaphase.

The irreversible mitotic arrest observed at the very low vincristine concentration of 4 ng/ml also suggests that induced mitotic arrest is responsible for the cytotoxic action in proliferating cells at clinically relevant vincristine concentrations. Serum concentrations of vincristine in patients have been reported by Bender et al. (2) to be 20 ng/ml or more and by Morasca et al. (13) to be 50 ng/ml or more within the first 2 hr after injection.

Mitotic arrest in NHIK 3025 cells exposed to 0.2 mM NY 3170 from 3 hr before mitosis is rather reversible when NCI 3170 is removed within 4 hr after entry into mitosis, as shown in a previous paper (19). This survival curve is redrawn in Chart 4 (dashed curve). However, when cells exposed to 0.2 mM NY 3170 from 3 hr before mitosis were exposed to 4 ng vincristine per ml in mitosis, lethal effects soon arose (Chart 4, Δ). Vincristine (4 ng/ml) alone added after entry into mitosis entailed little effect on NHIK 3025 cells (Chart 4, O). From this, one can conclude that there is a synergistic effect between NY 3170 and vincristine in mitosis with respect to cell inactivation. It seems that sublethal damage caused by NY 3170 in mitosis is manifested immediately as unrepairable damage by the presence of vincristine.

The main conclusions from this investigation are the answers to the 5 questions in the "Introduction." 1. Treatment with 4 ng vincristine per ml (4.3 nM) in G2 entails irreversible mitotic arrest in NHIK 3025 cells. This is well within the clinically relevant concentration range.

2. Mitotic arrest following vincristine treatment is primarily induced in late S and G2.

3. Lethal cell damage by vincristine in proliferating NHIK 3025 cells is also primarily induced in late S and G2. This indicates a correlation between the mitotic arrest properties and the inactivating effect of this drug.

4. The age-response curves of the mitotic inhibitors vincristine and NY 3170 both demonstrate drug resistance in G1, and increasing sensitivity throughout the cycle. However, mitotic cells are resistant to vincristine, while mitosis is the most sensitive stage with respect to inactivation by NY 3170.

5. During interphase, the combination of vincristine and NY 3170 yields survival values which equal the sum of the lethal effects from each drug alone. In mitosis, however, these 2 mitotic inhibitors demonstrate a synergistic effect. Mitotic arrest caused by vincristine is more irreversible in nature than arrest caused by NY 3170.

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