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# A Study of Factors Related to the Action of 1-Propargyl-5-chloropyrimidin-2-one (NY 3170) and Vincristine in Human Multicellular Spheroids

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**Abstract**—*Cells of the line NHIK* 3025, *originating from a human cervix carcinoma* in situ, *were protected against the lethal effects of the mitotic inhibitors* 1-*propargyl*-5-*chloropyrimidin*-2-*one* (*NY* 3170) *and vincristine when treated as multicellular spheroids* in vitro. *Cell survival was measured as retained colony-forming ability after* 24 *hr of treatments and the survival values of cells exposed in spheroids were* 10–100 *times higher than the survival values obtained by parallel treatments of exponentially growing monolayer populations of the same cell line. By exposure of spheroids to radioactively labelled drugs and subsequent autoradiography, it was verified that both NY* 3170 *and vincristine were able to penetrate the cell mass in the spheroid. Monolayer populations in the plateau phase of growth were also resistant and this suggests that the resistance of spheroids to NY* 3170 *and vincristine may be associated with a subpopulation of resting cells. The metaphase-arresting properties of both drugs were expressed in the spheroids, as an accumulation of cells in metaphase was observed following drug treatment.*

## INTRODUCTION

THE CULTURE of multicellular spheroids developed 10 years ago by Sutherland and coworkers[1] proved a useful model for radiobiologists[2] and in experimental chemotherapy, where selective inactivation of noncycling cells in the spheroid was observed by metronidazole [3]. More recently, the effects of several clinically effective anti-cancer drugs on spheroids from murine tumours have been established[4, 5]. However, most *in vitro* studies of drug effects on mammalian cells have used monolayer or suspension cultures. Since spheroids form a system of intermediate complexity between solid tumours and standard *in vitro* cultures[2], a comparison of drug effects in these two *in vitro* systems is of interest.

Drug resistance has been demonstrated in cells exposed as spheroids when compared to the response of single cells[6, 7]. This has been attributed to a reduction in drug delivery to the inner spheroid cells for adriamycin in V79 and EMT6 spheroids[7, 8], and for methotrexate in spheroids of human osteogenic sarcoma cells[9]. However, other factors, probably related to the metabolic state of these cells, have also been implicated[7]. The resistance of NHIK 3025 spheroids to vincristine could not be explained by a drug diffusion barrier, since cells throughout the spheroid were almost

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equally resistant to vincristine[10]. The present investigation was initiated to elucidate the mechanisms responsible for this resistance and the similar results obtained with NY 3170.

## MATERIALS AND METHODS

*Mitotic inhibitors*

NY 3170 (1-propargyl-5-chloropyrimidin-2-one, M.W. = 169) was synthesized at the University of Oslo, as published previously[11]. Before each experiment NY 3170 was dissolved in medium and the drug solution sterilized by Millipore filtration. [$^{14}$C]-labelled NY 3170 (specific activity 0.15 mCi/mmol) was kindly prepared by Professor S. G. Laland at the Department of Biochemistry, University of Oslo.

Stock solutions of vincristine (Eli Lilly & Co., IN, U.S.A.) in Hanks' solution were made weekly and stored at 4°C. Before each experiment the appropriate concentrations of vincristine in medium were prepared (1 ng/ml = 1.08 nM). [$^{3}$H]-labelled vincristine, with a specific activity of 3.45 Ci/mmol, was purchased from the Radiochemical Centre, Amersham, England.

*Cell culture*

The culture medium was E2a [12] containing 20% human serum and 10% horse serum. Stock cultures were grown in monolayer and trypsinized 3 times a week to maintain almost continuous exponential growth. In exponentially growing monolayer populations of NHIK 3025 cells the growth fraction is close to unity. The mean cell cycle time and the population doubling time is 18 hr[13, 14]. The plating efficiency of such cultures is 85–100%.

Plateau-phase monolayer populations were prepared by growing cells in culture flasks for 7 days without re-culturing, until a confluent cell layer with only minimal division activity had been obtained[10]. In this work the average plating efficiency of plateau-phase populations was 61% (S.E.M. = 12%).

Single-cell suspensions were obtained by seeding trypsinized cells in 25 cm$^2$ plastic culture flasks containing 12 ml medium. The flasks were then agitated on a tilting platform ("Rotary Mixer" from Cenco Inst., Breda, The Netherlands) with a speed of 50 periods per min. The population doubling time of NHIK 3025 cells in single-cell suspension is about 24 hr (Rogne, unpublished observations). The average plating efficiency after 24 hr in suspension was 59% (S.E.M. = 15%).

During the growth period and during drug exposure the spheroids were kept in plastic tissue culture flasks pre-coated with a thin layer of 1% agar. The mean spheroid diameter at the start of an experiment was 400–600 $\mu$m. Within each experiment the standard deviation of spheroid diameters was 60–70 $\mu$m [10]. In some experiments the spheroids were subjected to fractionated trypsinization [10] to obtain cell populations from different depths in the spheroid. In the periphery of NHIK 3025 spheroids, the growth fraction and the mean cell cycle time are about 0.65 and 30 hr respectively, as compared to 0.47 and 41 hr in the inner region, i.e., 150 $\mu$m from the spheroid surface [14]. The average plating efficiency of cells from spheroids was 62% (S.E.M. = 3%) and was independent of distance from the spheroid surface.

*Mitotic index measurements*

After a 6 hr drug exposure of exponentially growing monolayer cells or spheroids, the cells were harvested by trypsinization (fractionated trypsinization of spheroids) and transferred to centrifuge tubes. Equal amounts of ice-cold medium was added immediately. The medium-trypsin mixture was removed after centrifugation and the cells resuspended in fixative (methanol + acetic acid, 3:1). Cell smears were made on microscope slides before staining with aceto-orcein. The smears were counted blind for registration of the mitotic index (percentage of mitotic cells). At least 1000 cells were scored in each experimental group.

*Cell survival measurements*

After a 24 hr exposure to the respective drug concentrations, the drug-containing medium, which always contained some loose, metaphase-arrested cells, was transferred to a centrifuge tube. The cells were then trypsinized and the single-cell suspensions thus obtained added to the respective medium-containing centrifuge tubes. After centrifugation and subsequent removal of the medium-trypsin mixture, the cells were re-suspended in control medium and seeded in Petri dishes (5 replicate dishes) for measurement of colony-forming ability. For cells exposed in single-cell suspension, the treatment was terminated by removing the drug-containing medium after centrifugation. The cells were then seeded in Petri dishes, as for trypsinized populations.

The number of cells seeded per dish was determined with a haemacytometer. All recognizable cells were counted, including cells that from morphological judgments were already dead at the time of plating. Cell yield calculations showed that practically all cells

present when the drug exposure started were taken into account when estimating cell survival. The cell yield per unit volume was always low in the core of treated as well as untreated spheroids, since most of the space in the inner regions was occupied by a necrotic mass.

Colonies containing more than 40 cells after an incubation period of 10–12 days were scored as viable. Surviving fractions were calculated as the ratio between the average number of viable colonies and the number of cells seeded per dish.

*Autoradiographic measurements*

Spheroids were exposed for 2 hr to either 6 mM [$^{14}$C]-NY 3170 or 650 ng [$^{3}$H]-vincristine per ml. After exposure, the spheroids were immediately fixed in phosphate-buffered formalin, dehydrated and embedded in paraffin. Serial sections were made and transferred to microscope slides. The unstained spheroid sections were then coated with Ilford L4 photographic emulsion for autoradiography and stored in darkness at 4°C.

Some of the autoradiographs were developed after 1 week for measurement of grain density. By means of a microscope with an ocular grid system the number of grains per unit area at different levels in the spheroid could be estimated. Other autoradiographs were not developed until 5 months after coating with emulsion in order to obtain grains enough for making photomicrographs.

For estimations of the relative number of grains per cell, monolayer cells in the exponential or plateau phases of growth, cells in suspension and spheroids were exposed for 2 hr to either 6 mM [$^{14}$C]-NY 3170 or 460 ng [$^{3}$H]-vincristine per ml. After trypsinization and subsequent centrifugation, the cells were fixed and stained, cells smears prepared, and autoradiographs made as described above.

## RESULTS

The data presented in Fig. 1 show the degree of metaphase accumulation in spheroids and in exponentially growing monolayer cultures caused by a 6 hr exposure to NY 3170 or vincristine. The fact that no ana- or telophases were observed indicates that no cells were able to escape the metaphase arrest at these drug concentrations. The mitotic index in monolayer cultures was lower after exposure to NY 3170 (Fig. 1A) than after vincristine (Fig. 1C). This is probably due to a prolongation of interphase during treatment with NY 3170. Such prolongation from treatment with NY 3170 has been observed earlier in work with synchronized

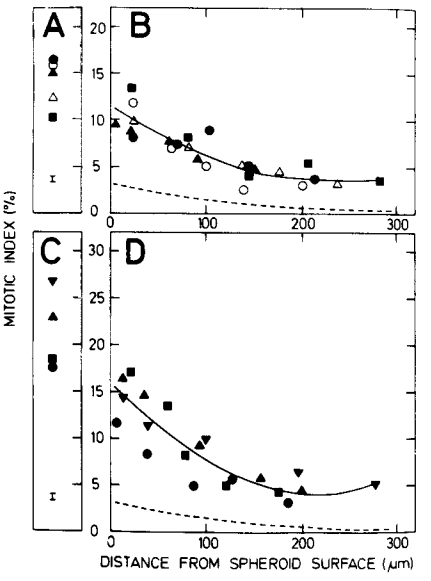


*Fig. 1. Mitotic index values in NHIK 3025 cell populations after a 6 hr exposure to NY 3170 (A and B) or vincristine (C and D). Exponentially growing monolayer cells (A) or spheroids (B) were exposed to 0.2 (open symbols) or 0.4 (closed symbols) mM NY 3170. The measured values for spheroid cell populations are plotted as a function of mean distance from the spheroid surface. Different symbols represent different experiments. The continuous curve in panel B was adapted by the method of least squares, taking all measured values into account. The vertical bar in A and the dashed curve in B show, for comparison, previously published mitotic index values in untreated cultures [14]. C and D show data from experiments parallel to A and B, but after exposure to 16 ng vincristine per ml.*

monolayer populations of NHIK 3025 cells [15]. Likewise, at all depths in the spheroid the mitotic index was somewhat lower after NY 3170 (Fig. 1B) than after vincristine (Fig. 1D). This indicates a slight prolongation of S or $G_2$ for cells exposed to NY 3170 while grown as spheroids, too.

Fractionated trypsinization of spheroids after 24 hr exposures to 0.4 or 2 mM NY 3170 demonstrated that the surviving fraction was virtually independent of distance from the spheroid surface (data not shown). This is in accordance with earlier published data for vincristine [10].

Figure 2 presents dose–response curves of monolayer cells in the exponential or plateau phases of growth, cells in suspension, and spheroids after 24 hr exposures to 0, 0.4, 1, 2 or 3 mM NY 3170. Cells exposed as spheroids were more resistant to NY 3170 than any of the other cell culture systems. Exponentially growing monolayer populations were more sensitive than plateau-phase populations. Most sensitive to inactivating effects of NY 3170 were, however, cells exposed in suspension. Complete disintegration eventually occurred for all cells exposed to 5 mM NY 3170 for 24 hr, including cells exposed as spheroids.

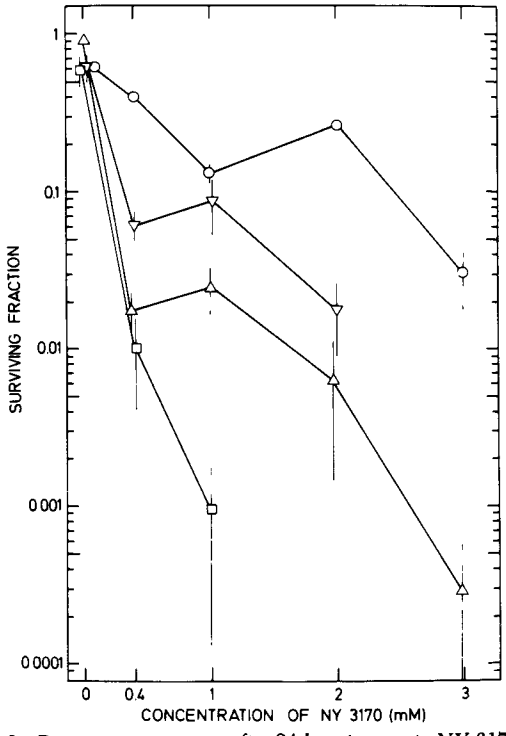


*Fig. 2. Dose–response curves after 24 hr exposures to NY 3170 of monolayer cells in exponential (△) or plateau (▽) phase of growth, cells in suspension (□) or spheroids (○). For each cell culture system, 3–7 independent experiments were performed. S.E.M. indicated as vertical bars.*

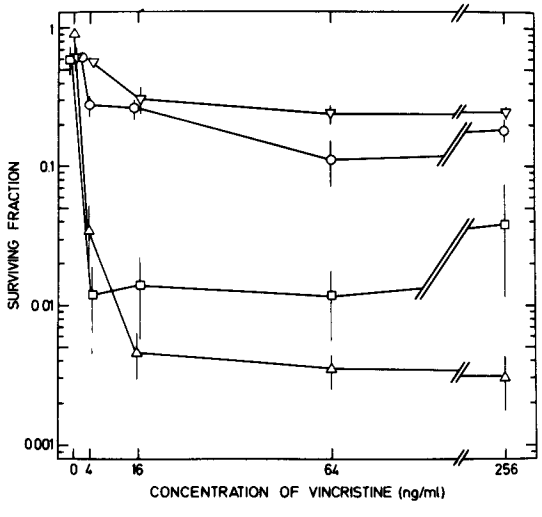


*Fig. 3. Dose–response curves after 24 hr exposures to vincristine of monolayer cells in exponential (△) or plateau (▽) phase of growth, cells in suspension (□) or spheroids (○). For each cell culture system, 2–5 independent experiments were performed. S.E.M. indicated as vertical bars.*

The dose–response curves presented in Fig. 3 were obtained by exposing for 24 hr NHIK 3025 cells to vincristine. Plateau-phase cells and spheroids were far more resistant to vincristine than exponentially growing monolayer cells and cells exposed in suspension. It is noteworthy that total disintegration was not observed in any culture, even after exposure to the very high vincristine concentration of 256 ng/ml.

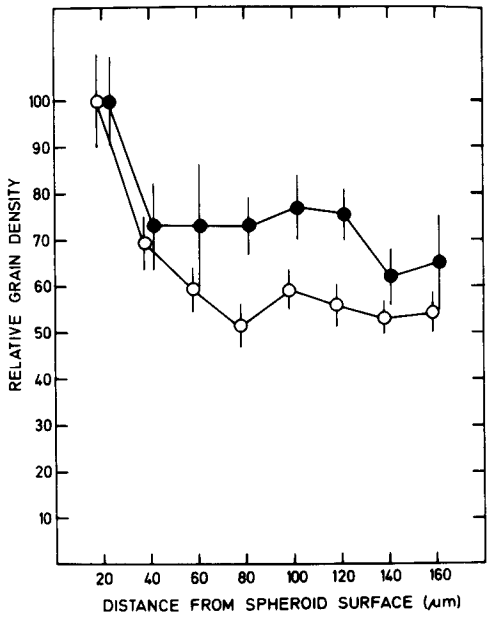


*Fig. 4. Drug distribution spheroids measured as the relative number of grains per unit area at different depths in central section autoradiographs after a 2 hr exposure of the spheroids to 6 mM [$^{14}C$]-NY 3170 (○) or 650 ng [$^{3}H$]-vincristine per ml (●). The plotted value at each depth is the mean of 10 estimations. S.E.M. indicated as vertical bars.*

Grain density measurements at different levels in the spheroid were performed on autoradiographs made after exposures to radioactively labelled drugs (Fig. 4). Levels of both vincristine and NY 3170 were highest at the spheroid periphery, but throughout the internal regions the drug levels never fell below 50% of these maximum values.

The spheroid section autoradiographs that were stored for 5 months in darkness before being developed revealed that [$^{14}$C]-NY 3170 was mainly associated with the cell nucleus. Most cells were heavily labelled, but some cells contained only trifling amounts of labelled NY 3170. The autoradiographs from [$^{3}$H]-vincristine-exposed spheroids demonstrated that this drug was more evenly distributed among the individual cells.

Figure 5 shows data on the amount of accumulated and bound drug per cell. In order to obtain an appropriate number of grains per cell, the cells were exposed to somewhat higher drug concentrations than used in the viability experiments. On the other hand, to avoid influence on the results from drug-induced cell-cycle kinetic perturbations, the exposure time was kept as short as 2 hr. The differences between the model systems as concerns cell viability were not reflected in the grain counts, except for a significantly lower amount of bound drug in plateau-phase monolayer cells.

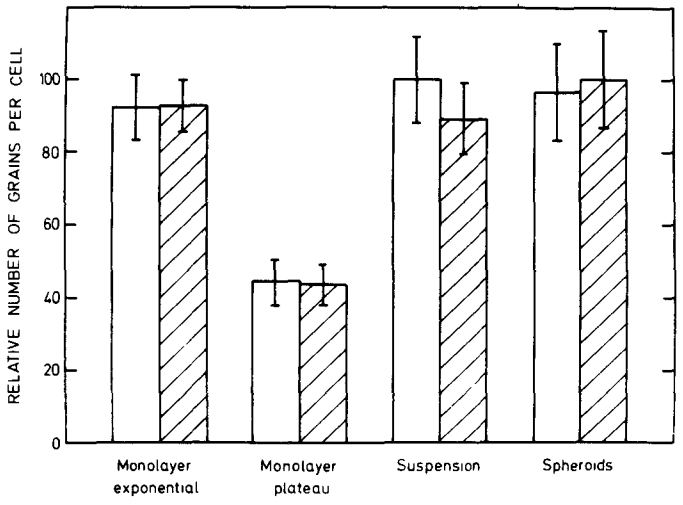


*Fig. 5. Relative number of grains per cell in autoradiographs made after exposures for* 2 hr *to* 6 mM [$^{14}C$]-*NY* 3170 (*open columns*) *or* 460 ng [$^{3}H$]-*vincristine per* ml (*hatched columns*). *In each experimental group* 50 *cells were taken into account. S.E.M. are indicated.*

## DISCUSSION

Both NY 3170 and vincristine were effective as metaphase-arresting agents in spheroids. The extent of accumulation was lower in spheroids, especially with increasing distance from the surface, than in exponentially growing monolayer populations (Fig. 1). There is every indication that this decrease in mitotic index was due to changes in cell kinetic parameters [14]. A similar decrease in mitotic index from the periphery to the centre of the tumour has been observed after *in vivo* exposure of mice bearing solid tumours to vincristine [16], and this was also associated with a decline of the growth fraction towards the tumour centre together with a diminished vascular supply.

The data in Figs. 2 and 3 demonstrate that spheroids were remarkably more resistant to the lethal effects of the drugs than exponentially growing monolayer cultures. This resistance appeared unlikely to be related to the rounded shape of the individual cell and/or the lack of attachment to a firm surface, since cells in suspension were susceptible to the lethal effects of both drugs.

In accordance with the present results, Bhuyan and co-workers [17], as well as Hill and Whelan [18], in work with L1210 mouse leukemic cells and NIL8 Syrian hamster ovary cells respectively, demonstrated that vincristine was more lethal to exponentially growing cells than to plateau-phase cells. On the other hand, Olah *et al.* [19] reported that plateau-phase Chinese hamster cells were more sensitive to vincristine than exponentially growing cells.

The resistance of plateau-phase NHIK 3025 cells to NY 3170 and vincristine suggests that the resistance in spheroids to these drugs were related to the presence of quiescent cells in the spheroids. In the inner regions of NHIK 3025 spheroids the growth fraction is about 50% [14]. The average plating efficiency of cells grown in spheroids was, however, 62% and was independent of distance from the spheroid surface. These results strongly suggest the presence of quiescent, but still clonogenic, cells in NHIK 3025 spheroids.

There might also have been, at least after NY 3170, a slight protection of inner spheroid cells due to a somewhat reduced drug concentration in this region. After 0.4 mM NY 3170, the surviving fractions were slightly higher for internal cells than for the outer spheroid cells. However, no distinct diffusion barrier could be observed for NY 3170 or vincristine.

It is noteworthy that we could not detect a significant decrease in survival in any of the culture systems when raising the vincristine concentration above 16 ng per ml (Fig. 3). When vincristine is used clinically, the initial concentrations of this drug in serum are usually above 70 ng per ml, but decrease rapidly with time [20–22]. The present results indicate that very high vincristine concentrations in the tumour for several hours, if achievable, might not automatically lead to better treatment results. The same lack of enhanced cell inactivation after 24 hr exposures to very high concentrations of vincristine was recently reported by Hill and Whelan [18] from work with NIL 8 hamster cells.

As concerns vincristine, Bleyer *et al.* [23] and Skovsgaard [24], both in work with mouse cell lines, demonstrated reduced uptake of [$^{3}$H]-vincristine in cells resistant to this drug. The

resistance to vincristine was also associated with enhanced drug efflux [24]. The data presented in Fig. 5, and the autoradiographs made from central sections of spheroids exposed to labelled vincristine, did not yield any evidence in support of the hypothesis that the resistance to vincristine in spheroids was due to an impaired accumulation and binding of drug within the cell.

As observed for vincristine, the mean number of grains per cell in spheroids and exponentially growing monolayer cells were identical after treatment with [$^{14}$C]-NY 3170. However, a small fraction of cells within the spheroid seemed to contain only trifling amounts of bound [$^{14}$C]-NY 3170 when compared to the majority of the cells. This might imply that the resistance to NY 3170 in spheroids was, to some extent, associated with a reduced intracellular drug concentration.

Finally, one can not exclude the possibility that the protection against NY 3170 and vincristine was partly related to the three-dimensional intercellular contact in spheroids.

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