EXCELLENT PUBLISHERS

International Journal of Current Research and Academic Review

ISSN: 2347-3215 Volume 4 Number 2 (February-2016) pp. 9-17

Journal home page: http://www.ijcrar.com

doi: http://dx.doi.org/10.20546/ijcrar.2016.402.002

Avinca alkaloid effect on microtubulues of H_{22} (Hepatic cell line)

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KEYWORDS

Cytoskeleton, Microtubule disruption, Cytoskeleton recovery, Vincristine

A B S T R A C T

The study evaluated the effect of selected Vinca alkaloids on the microtubule network of H_{22} cells and the recovery of its disruption. Cells were treated with Vincristine at various concentrations from 2 µg/l to 800 µg/l for 60 min, or with Vincristine at a concentration of 4615 µg/l and 9230 µg/l for 60 min. Microtubules were detected by means of indirect immunofluorescence. The damage was examined in a fluorescence microscope. Also, cells were treated for 60 min with vinblastine at concentrations of 2 µg/l or 800 µg/l for 60 min, and the recovery process was studied in time intervals of 6, 7, 8, 9, 10 hours, or 12 hours, respectively. Differences in the arrangement of microtubules were assessed by means of a software for quantification of the cytoskeleton changes in cells treated with Vincristine at a concentration of 20 µg/l and in untreated control cells.

Introduction

In eukaryotic cells the cytoskeleton is formed by three major structural elements– microtubules, microfilaments, and intermediate filaments (1). The cytoskeleton plays a specific role in cell division, maintenance and changes of cell 20 shape, in intracellular contacts, interaction with membranes, extracellular matrix, and in cell motions. Microtubules are filaments with the largest diameter of all cytoskeletal components. Microtubular network is important for the execution of many cell functions. They play an important role in cell division. The microtubular diameter measures about 25 nm.

Microtubules are composed of 13 equally spaced protofilaments (2). Tubulin is the basic protein of the microtubules and molecules of tubulin are arranged in dimers containing both its forms: α-tubulin and β-tubulin. Microtubules are continuously changeable structures (3) and polymerisation and depolymerisation of MTs is regulated by extracellular and intracellular factors (4). The presence of GTP at microtubule ends is necessary to maintain the stability of the polymer (11). The cytoskeleton can be damaged through the effect of many external factors or chemical agents (5–10). The

opposite ends of free microtubules show different sensitivities to microtubule depolymerising agents such as low temperature, Ca++ or colchicines (12). The mitotic spindle is a self-organising structure that is constructed primarily from microtubules. Among the most important spindle microtubules are those that bind to kinetochores and form the fibres along which chromosomes move. Vinca alkaloids – vincristine and vinblastine – are microtubular toxins of chemically similar nature (13) that disrupt microtubule function by binding to a site on β-tubulin and suppressing microtubule dynamics. Although they are closely related in physical and chemical properties, they have various effects on the human body. Vinca alkaloids perturb the kinetochore-microtubule attachment.

This activates a checkpoint pathway that ensures proper attachment of chromosomes to the mitotic spindle (14–16): When microtubules fail to attach to one or more kinetochores as a result of drug treatment, the checkpoint components continue to generate signals that inhibit the metaphase/anaphase transition that delays cell cycle progression and induces programmed cell death (17). At higher drug concentrations, vinca alkaloids induce the assembly of spiral filaments of tubulin, which, consequently, can interact laterally and form para crystals (18).

This action is similar to the action of rganizatio, but is different from that of paclitaxel, which promotes the polymerization of tubulin polymers to form abnormal stable microtubule structures. Treatment with high concentrations (100 nM) of vincristine cause disruption of microtubule organization, which, in turn, prevent accumulation of p53 in the nucleus. By contrast, treatment with lower drug concentrations (3 nM) that are known to suppress microtubule dynamics but do not alter microtubule organization enhance nuclear accumulation of p53 and its downstream transactivated elements above physiological levels (19). Vincristine and vinblastine have been widely used to treat cancer (e.g. acuteleukaemia, rhabdomyosarcoma, neuroblastoma, Hodgkin's disease), to synchronise cell cycle, or to look for defects in the mitotic checkpoint (13).

Materials and Methods

Cell line

H22 cell line) (from the Department of Biology, Faculty of Medicine, wuhan University - china) were grown in monolayers at 37°C without antibiotics in 5%C02/95% air (7). Cell proliferation was determined by countingcells by hemocytometer at the time of alkaloids addition and20 h later. Mitotic index, cell morphology, and spindle interpolar distances were determined by immunofluorescence microscopy (8).

Exposure to vincristine and vinblastine

In the first series of experiments, to investigate the action of Vincristine and Vinblastine onto the microtubule network, a solution containing 1 mg of Vincristini sulfas in 1 ml of medium (Vincristine-Teva, inj.), or an original solution containing 5 mg of Vinblastini sulfas in 5 ml of medium (Gedeon Richter), was mixed with 3 ml of growth medium in each of the Petri dishes so that the final concentration of Vincristine, or Vinblastine was 2, 10, 20, 30, 40, 80, 100, 200, 400, 800 μg/l, or 4615 μg/l and 9230 μg/l (Vincristine only). Each concentration was in two dishes. The cells were exposed to the drugs for 60 min at 37 °C. After the

treatment, the samples were washed three times for 4 minutes concurrently with control samples in phosphate-buffered saline (PBS, pH 6.9) and processed for immunofluorescence microscopy.

In the second series of experiments the cells were cultivated for 2, , 10, 20, 30,and 60 minutes at 37 °C in media with a final Vincristine concentration of 20 µg/l, or for 5 minutes in a medium containing Vincristine at a concentration of 800 µg/l. The samples were washed three times for 4 minutes concurrently with control samples in PBS, pH 6.9, and processed for immunofluorescence microscopy. The third series of experiments was performed with vincristine at a final concentration of 20 µg/l. The cells were exposed to the drug for 60 min. After the treatment, the drug-containing medium was poured off and monolayers were subjected to three washing procedures concurrently with control samples with phosphate-buffered saline (PBS) (pH 6.9).

Two slips with the cells were then subjected immediately to fixation and detection of the microtubular network as well as two slips which were cultivated for 60 min in vincristine-free growth medium as a control. The other Petri dishes were refilled with fresh growth medium and incubated for another 6, 7, 8, 9, and 10 hours in order to evaluate the recovery processes. Recovery progressed at 37 °C in a humidified atmosphere of 95 % air and 5 % CO2. The other control monolayers treated with a growth medium only containing vincristine-free DMEM were cultured in the same conditions as the vincristine-treated cells recovered for 10 hours. After fixation, the microtubular components were visualised and viewed in a fluorescence microscope. A similar attempt was provided for a vincristine concentration of 400 µg/l. The cells were exposed 22 to the drug for 60 min, and after the washing procedure, they were recovered for 8 or 12 hours in the same condition as last mentioned. Microtubular components were visualised and examined in a fluorescence microscope. To quantify the cytoskeleton changes, cells were incubated in a fourth series of experiments at 37°C in a medium containing vincristine at a concentration of 20 µg/l for 60 minutes. They were cultivated on two coverslips in two Petri dishes. The control cells on two slips were cultivated parallelly. The medium was poured off and the cells were subjected to three washing procedures with PBS (pH 6.9) and fixation.

Results and Discussion

Untreated control cells (Fig. 1) showed a microtubule network regularly distributed along the whole cell content. Cells exposed to drugs at concentrations of 2 µg/l for 60 min did not show considerable changes in the distribution of microtubules. Cells exposed to Vincristine or Vinblastine at concentrations of 10 (Figs. 2, 3), 20, 30, 40, 80, 100, 200, 400, and 800 µg/l for 60 min showed changes in the arrangement of the microtubular network. The network of cytoplasmatic microtubules at concentrations of 10, 20 µg/l was thinned down, and individual fibres had a wavelike shape. The network damage increased with increasing concentration of cytostatics. The microtubules were more thinned down and fragmentation of fibres occurred. At a higher concentration of 400 µg/l, sometimes blebs were formed (Fig. 4). Cells exposed to vincristine at concentrations of 4615 µg/l and 9230 µg/l formed paracrystals (Fig. 7, 8). No significant difference was detected in vincristine and vinblastine treated cells. When cells were exposed to vincristine at a concentration of 20 µg/l for 2, 5, or10 minutes, no noticeable changes occurred in

the microtubule network. The 20-min treatment at a concentration of 20 µg/l caused disruption of microtubules. The network was thinned down, and individual fibres had a wavelike shape. The cells exposedto vincristine at a concentration of 800 µg/l for 5 minutes showed a severely defective microtubular network.

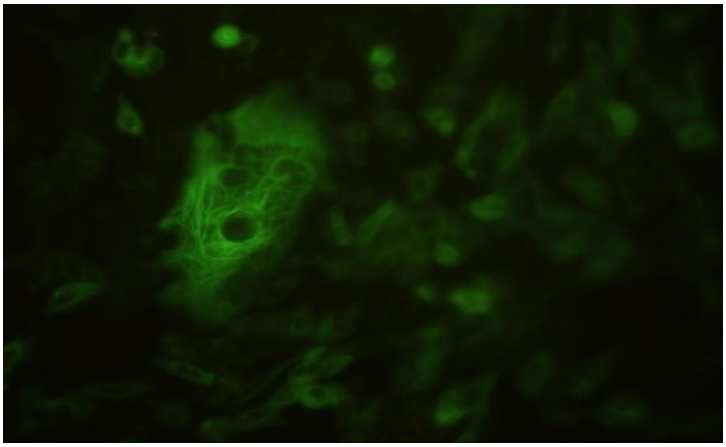

Fig.1 Microtubules of untreated control cells of H_{22}cell line. The network is regularly distributed along the whole cell content

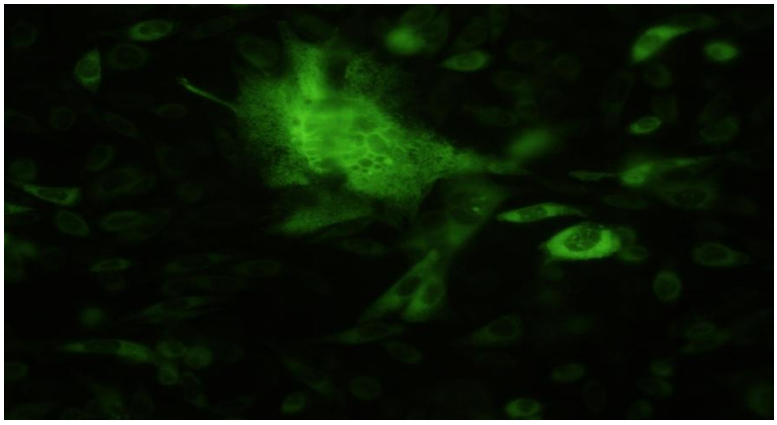

Fig.2 Microtubules of H_{22} cells treated with vinblastine at a concentration of 10 µ/l for 60 min. The network of cytoplasmatic microtubules is thinned down, and ndividual fibres have a wavelike shape.

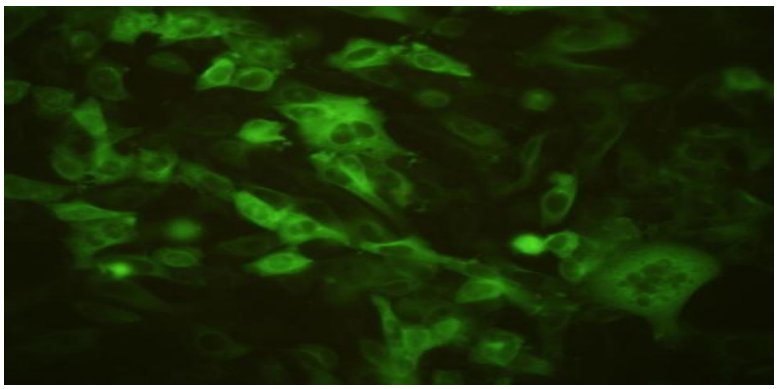

Fig.3 Microtubules of H_{22} line cells treated with vincristine at a concentration of 10 μ/l for 60 min. The network of cytoplasmatic microtubules is thinned down, and individual fibres have a wavelike shape.

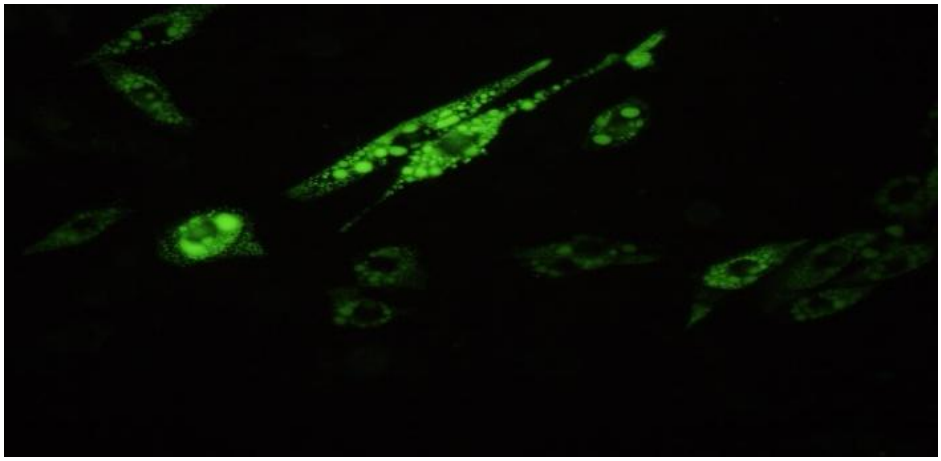

Fig.4 Cells of H_{22} line treated with vincristine at a concentration of 400 μg/l for 60 min. There are blebs formed on the periphery of the cells. The microtubules perished and free tubulin was detected along the whole cell content.

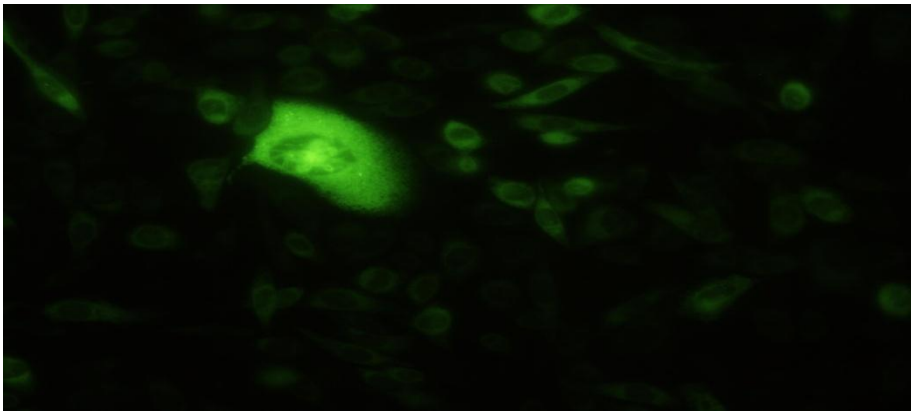

Fig. 5 Microtubules of H_{22} line cells treated with vincristine at a concentration of 400 μ/l for 60 min.Microtubules recovered for 8 hours. The network is partially

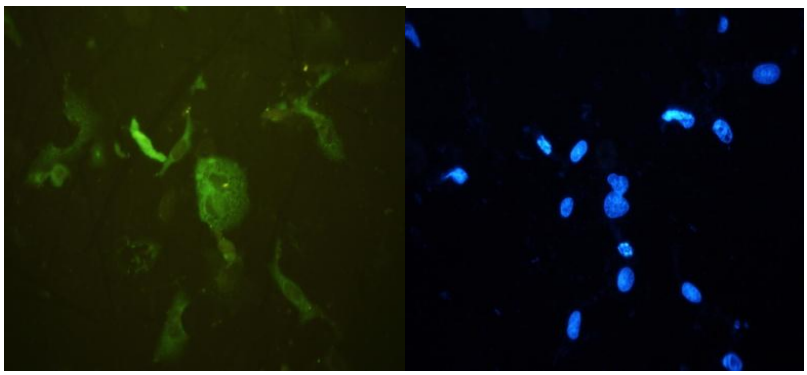

Fig.6 Microtubules of H_{22} line cells treated with vincristine at a concentration of 400 μ/l for 60 min. Microtubules recovered for 12 hours. The network is nearly restored.

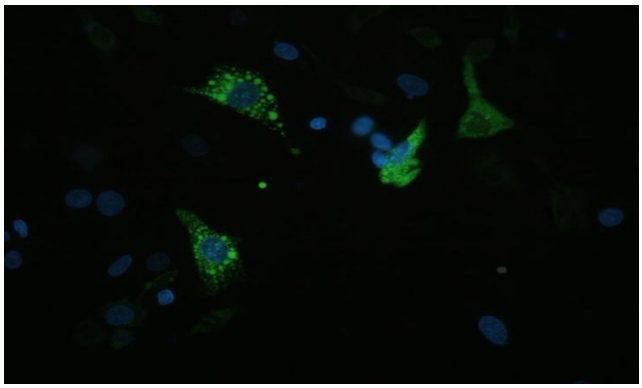

Fig.7 H_{22} cells treated with vincristine at a concentration of 4615 μg/l for 60 min. Paracrystals are formed

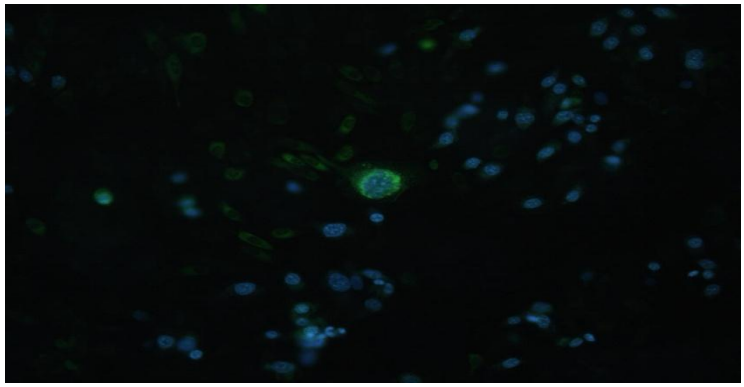

Fig.8 H_{22} cells treated with vincristine at a concentration of 9230 μg/l for 60 min. Paracrystals are formed.

All the cells with the recovering period of 6 hours in a drug-free growth medium following vincristine treatment showed damage of microtubules. The cells after a 7-hour recovery period had their microtubular network either fully restored or still damaged. After recovery for an 8-hour period, some cells showed a partly defective(thinned-down) network, but the majority of the cells showed restored microtubules. When the cells were allowed to recover for 9 hours, the microtubules were spread out comparably to those observed in untreated control cells. The control cells showed their microtubule network regularly distributed along the whole cell volume. When cells were recovered after treatment with vincristine at a concentration of 400 μg/l for 8 hours, the cytoskeleton was partially restored afterwards (Fig. 5).

The microtubules were thinned down, and individual fibres had a wavelike shape. After a recovery period of 12 hours, the network was also damaged (Fig. 6),only several cells showed nearly restored microtubules.In a fourth series of experiments the results of microtubule structure measurements were statistically analysed. The data obtained

from a slip constituted a group. The groups obtained from the two slips treated with vincristine at a concentration of 20 µg/l were compared with one another. The observations are demonstrated by a box plot (Fig. 9). The values obtained from the two slips were homogeneous (consistency in medians and in distributions) and therefore were summed up for the final analysis. The groups obtained from both control slips were compared in the same manner (Fig. 10) and summed up for final statistical evaluation, because they were homogeneous. Further, we compared the control and vincristine-treated groups (Fig. 11). The hypothesis about consistency in medians was rejected (P=0.00006) and the hypothesis about consistency in distributions was rejected, too (P=0.0003). The decrease in the quantity of microtubules in the cytoplasm was significant in vincristine-treated cells.To quantify cytoskeleton changes during the recovery processes, we summed up all data obtained from the binate slips with the cells recovered for a particular period (6, 7, 8, 9, 10 hours) and from slips with non-recovered cells (Figs. 12, 13). Multiple comparisons of medians were performed (Tab. 1). There were significant differences in non-recovered cells and all other groups (recovery periods of 6, 7, 8, 9, and 10 hours). The quantity of microtubules in the cytoplasm was significantly higher ($P<0.05$) in cells with a recovery period lasting from 6 to 10 hours than in the non-recovered cells. The results prove recovery processes and restoration of the microtubular cytoskeleton.

The interaction of antitumour agents with compounds of the cytoskeleton is a theme studied in many papers (5–8, 9, 7, 18, 21–24). Lobert (22) studied the interaction of vinca alkaloids with tubulin, and compared vinblastine and vincristine. She studied e.g. the affinity of the drug for tubulin heterodimers. Vincristine exhibited a higher overall affinity for porcine brain tubulin than vinblastine, but the affinity of the drug for tubulin heterodimers was identical for the two drugs. Under our experimental conditions we did not mark any differences between the two drugs. Some authors also studied the recovering processes of the cytoskeleton after treatment of cell cultures with physical factors or agents interfering with cytoskeleton compounds. Vincristine caused a sequence of morphological changes in sensitive cells from three pleiotropic resistant MCF-7 human breast carcinoma cell lines mixed with vaginal adenocarcinoma cells. The cells were selected in serially increasing drug concentrations. These changes included precipitation of tubulin and disappearance of tubular structure. The changes occurred initially within 3 hours of incubation, but were expressed in all cells after 6 hours. If, after 3 hours of drug exposure, the cells were subcultured in drug-free media, the cytoskeletal structure reformed within 10 hours.

The maximal recovery of the cytoskeletal structure occurred 22 hours after drug removal and was sustained up to 36 hours (23). Treatment with vincristine (24) eliminated the microtubule bundles, leaving only tubulin paracrystals. Within 24 hours after washing out the vincristine, the microtubule bundles repolymerised in cultured hippocampal neurons**.**

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How to cite this article:

Ibrahim Hadi Mohammed. 2016. Avinca alkaloid effect on microtubulues of H22 (Hepatic cell line). *Int.J.Curr.Res.Aca.Rev.* 2016.4(2): 9-17. **doi:** http://dx.doi.org/10.20546/ijcrar.2016.402.002