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IN VITRO CYTOTOXICITY OF POLYINDOLENINE ALKALOIDS ON RAT HEPATOMA CELL LINES. STRUCTURE ACTIVITY RELATIONSHIPS

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Summary

New groups of polyindolenine alkaloids have been isolated from new species of the tribe Psychotriae (Rubiaceae). The cyto-inhibitory effects of these compounds on tumoral cell lines have been thoroughly investigated and thus allow the statement of relationships between some original structural patterns and the observed biological effects.

Introduction

Ethnopharmacological investigations in the Pacific Islands led to the discovery of new species of the genus *Psychotria* (tribe Psychotriae, Rubiaceae). In the family Rubiaceae, only a few members of the genus *Psychotria* (*P. viridis*, *P. carthaginensis*) have been used in hallucinogenic preparations. The traditional uses of *Psychotria* are very different from one geographical region to another. Among the five species of *Psychotria* (*P. aneityensis*, *P. nacdado*, *P. trichostoma*, *P. milnei*, *P. forsteriana*) encountered in Vanuatu, three contain alkaloids while most of the *Psychotria* studied so far do not. The chemical study of *Psychotria* species from New Caledonia (*P. oleoides*) and Vanuatu (*P. forsteriana*) led to the isolation of alkaloids of the polyindolinic type.

Polyindolenine alkaloids have been isolated from plants of the Rubiaceae family from different geographic origins (New Guinea, Australia, New Caledonia). They are polymers consisting of three to five tryptamine units and

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were shown to be very cytotoxic on tumoral cell lines: on HTC (Roth et al., 1986) and slightly on L_{1210} cells (Libot, 1982). These characteristics made Roth et al. consider them as potent cytotoxic antitumoral agents (Roth, 1986). Their activities are dose-dependent and vary according to their structures (Roth, 1986).

Numerous possibilities of isomerism exist among these structures, on account of their mode of polymerisation and of their great number of assymetric carbons.

The isolation of new groups of such compounds from new plant species, containing up to eight units (Saad, 1986) incited us to study further their biological properties and the relationships between the structural features and the activity of these molecules.

Materials and methods

Extraction, isolation and identification of the alkaloids have been described elsewhere in other communications (Roth, 1985,1986; Hart, 1974).

Antitumor agents

Colchicine was obtained from Rhone Poulenc Laboratories, vinblastine sulphate (Velbé®) from E. Lilly Laboratories, methotrexate from Specia Laboratories, 5-fluoro uracil from Roche Laboratories and doxorubicine from Roger Bellon Laboratories.

Cytotoxic tests in vitro

Cytotoxicity tests were performed on cultured rat hepatoma cells (HTC line) derived from clone 7288 of a Morris rat hepatoma. The cells were cultured in suspension, in Swim's S-77 medium complemented with 10% neonatal calf serum (GIBCO). The cells were incubated in 75-ml culture flasks at 37 °C, under magnetic stirring. At the start, the cultures were diluted with fresh medium and adjusted to 10^5 cells per ml. Under these conditions, the cell growth was exponential during three to four days in the same medium, with an average generation time of 28 h, before reaching the stationary phase at a density of about 8×10^5 cells per ml. The test compounds were dissolved in ethanol and added to each flask to give various final concentrations. The amount of ethanol was adjusted to give final concentration of 0,1% in all cases including control cultures. Control experiments showed that this amount of ethanol (0.1% v/v) had no effect on cell proliferation. Cell growth was measured every 24 h, by counting the number of the cells with a Neubauer microcytometer. Samples of culture were previously incubated for 15 min in the presence of Trypan Blue (10%), in order to estimate the number of viable and dead cells.

The viability of the cells was expressed as a function of time, by means of growth or survival curves. The comparison of the different viability curves with that of a standard was used to determine the biological activity of the tested compounds, at a given concentration (inactive, cytotoxic).

Results and discussion

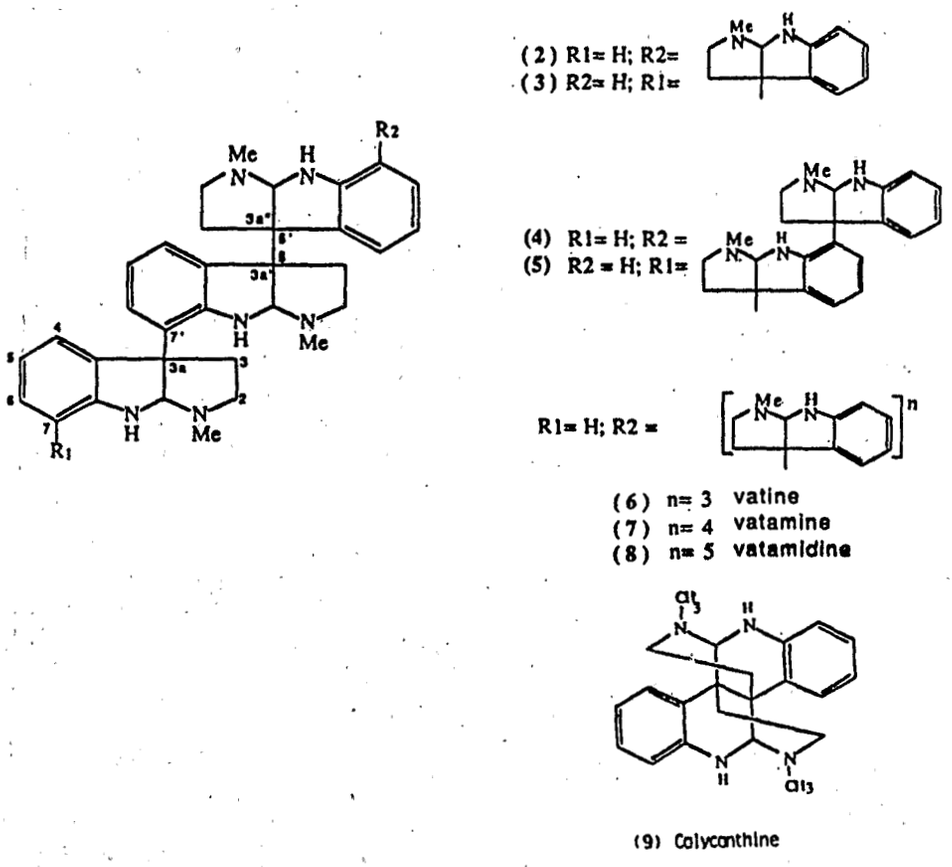
This work is a part of a large research programm concerning the Pacific Islands flora, undertaken in collaboration with the "ORSTOM" (Office de la Recherche Scientifique et Technique Outre-Mer), in order to evaluate the medicinal plants of these islands. These studies led to the discovery of plants containing new compounds with original structures. Polyindolenine alkaloids have been found in various plants: *Psychotria oleoides* from New Caledonia (Libot, 1982), *Psychotria beccaroides* from New Guinea (Hart et al., 1974), *Psychotria forsteriana* (Roth et al., 1985) and *Calycodendron milnei* from Vanuatu (Saad, 1985). All belong to the same tribe (Psychotrieae) of the family Rubiaceae. Similar polymers have also been found in *Hodgkinsonia frutescens* (Fridrichsons et al., 1967), also Rubiaceae, from Australia, but belonging to the tribe Chiococceae. As reported before, these alkaloids presented interesting chemical as well as pharmacological properties (Roth et al., 1986; Libot et al., 1987; Parry et al., 1978). Polyindolenine alkaloids containing six, seven and eight tryptamine units have recently been isolated and described (Saad, 1986).

In order to determine the relationships between the structures and activities of these compounds, we chose among the major alkaloids isolated, some compounds belonging to different groups. Using the various original structural patterns readily present in the plant, we could investigate the influence of the following parameters on the cytotoxicity of these compounds to rat hepatoma cells (HTC): molecular weight, structural isomerism, stereoisomerism and mode of polymerisation. Moreover, the cytotoxic activity of these alkaloids was compared to that of some well known antitumor agents.

This study includes the following compounds (Scheme I), which have been isolated mainly from *Calycodendron milnei*, a new species endemic in Vanuatu and from *Psychotria forsteriana*. They are all derived by the polymerisation of tryptamine units: hodgkinsine (H) (1) and its stereoisomer, hodgkinsine A (Ha); quadrigemine G (Qg) (2) and H (Qh) (3); psychotridine C (Pc) (4), isopsychotridine D (Id) (5) and E (Ie); vatine (V) (6) and its stereoisomer vatine A (Va); vatamine (Vt) (7); vatamidine (Vm) (8); and lastly calycanthine (cal) (9), structurally different, but closely related to the others by its biosynthetic pathway (Hall, 1967).

The main differences between these various alkaloids are consequently their molecular weight and their number of basic units. As discussed by Saad (1986), the trimers H and Ha are stereomers.

Alkaloids Qg and Qh are isomers of four units but in which the C_{3a} to $C_{3a'}$ bond (= $\beta-\beta'$ bond involving two aliphatic carbons in the β position of the nitrogen) links a group of two units in the case of alkaloid Qg (quadrigemine A type) whereas in alkaloid Qh the $\beta-\beta'$ bond links one unit to a group of three such units (quadrigemine B type). Compounds Id and Ie are also based on the same model, in which one terminal unit is linked to a group of four by the $\beta-\beta'$ bond. The other compounds are based on the quadrigemine A type



Scheme 1. Polyindolenine alkaloids: main structural patterns (excluding stereochemistry). (1) hodgkinsine type, H, Ha; (2) quadrigemine A type, Qg; (3) quadrigemine B type, Qh; (4) psychotridine type, Pc; (5) isopsychotridine type, Id, Ie; (6) vatine type, V, Va; (7) vatamine, Vt; (8) vatamidine, Vm; (9) calycanthine, cal.

model, i.e., the $\beta-\beta'$ bond links two terminal units to a group of three, four, five or six units respectively for the pentamer (Pc), the hexamers (V, Va), the heptamer (Vt) and the octamer (Vm). The exception is calycanthine (9), a tetrahydroquinoline dimer in which this type of bond does not occur.

The cytotoxic effects are expressed as a function of time by means of growth or survival curves.

Effect of dose

The different alkaloids at various concentrations were added to the culture media during 72 h. According to the viability curves, the hodgkinsines, H, Ha (Fig. 1a) at 8×10^{-6} M are extremely toxic with 100%

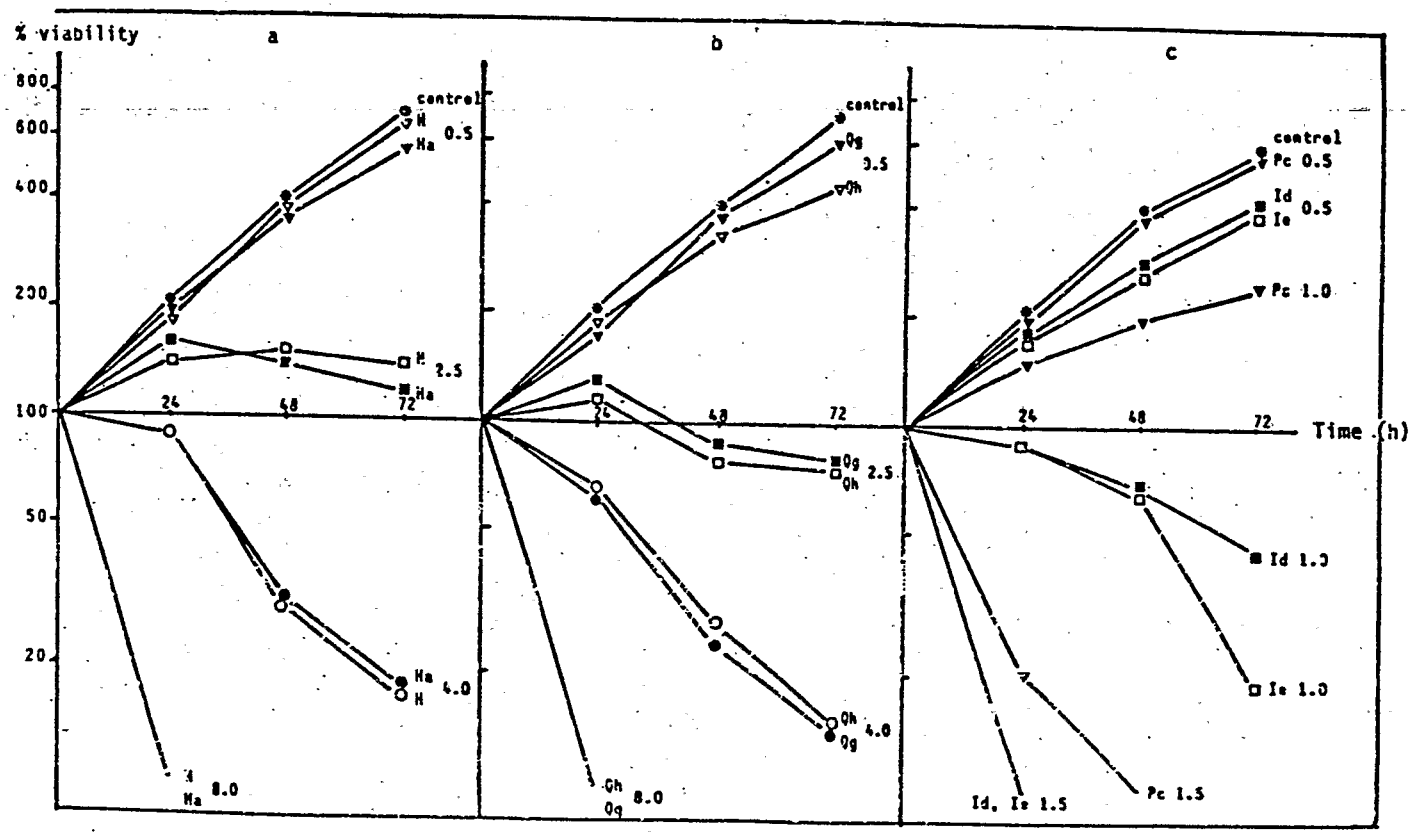


Fig. 1. In vitro dose-dependent cytotoxic effect of polyindolenine alkaloids on HTC cells (concentrations in μM). (a) Trimers: H, hodgkinsine; Ha, hodgkinsine A. (b) Tetramers: Qg, quadrigemine G; Qh, quadrigemine H. (c) Pentamers: Pc, psychotridine C; Id, isopsychotridine D; Ie, isopsychotridine E.

cellular mortality after 24 h. They are slightly toxic at 4×10^{-6} M with 10% mortality after 24 h but this toxicity increases with the time of contact during which 80% mortality was reached after 72 h. The quadrigemines (Fig. 1b) slightly inhibit cell proliferation at 0.5×10^{-6} M but respective cellular mortality rates of 100% and 35% were obtained at 8×10^{-6} M and 4×10^{-6} M after 24 h. This toxicity increases as a function of time with about 90% mortality after 72 h. In Fig. 1c, the isopsychotridines D and E are more toxic than psychotridine C.

Vatine (Va), vatamine (Vt) and vatamidine (Vm) are much more cytotoxic than the other alkaloids. According to Fig. 2, they are either inactive or extremely cytotoxic without showing an intermediary growth inhibition state as seen with the above mentioned alkaloids. In this case also, toxicity increases with the increase of incubation time.

It appears that the cytotoxic properties of polyindolenine alkaloids are dose-dependent and increase with increasing time of incubation, the alkaloids with higher molecular weight being the most cytotoxic.

Effect of molecular weight

Figure 3 represents the viability curves of HTC cells incubated with the different alkaloids of increasing number of tryptamine units at the same concentration (10^{-6} M) during 72 h. It appears that the cytotoxicity of these compounds is a function of their molecular weight after 24 h. Isopsychotridines D and E are less toxic with about 30% mortality after 48 h, increased to 55% or 80% according to the alkaloids after 72 h. Psychotridine C at this concentration leads only to growth inhibition without killing the cells. Hodgkinsine and quadrigemines are the least active. It seems that alkaloids with more than five basic units exhibit very important cytotoxic activity on the HTC cell line. It is also of interest to note that the activity increases with incubation time.

Effect of structural isomerism

As mentioned earlier, psychotridine C (Pc) and isopsychotridine D (Id) have five units each but in psychotridine a group of two units is linked to a group of three by a $\beta-\beta'$ bond while in isopsychotridine the $\beta-\beta'$ bond links a group of four units to a single residual one. Viability curves of HTC cells incubated with these different isomers at concentrations of 10^{-6} M and 1.5×10^{-6} M (in Figs. 1c and 3) show that isopsychotridines D and E are more cytotoxic than psychotridine C with 100% mortality after 24 h at 1.5×10^{-6} M. Pentamerous alkaloids with a single terminal moiety seem more active than those with two terminal units.

Effect of stereomerism

Four pairs of stereomers were chosen. Hodgkinsine and hodgkinsine A, quadrigemine G and quadrigemine H, and isopsychotridines D and E. From Figs. 1a, 1b, 1c, one can observe that each pair of stereomers, at the same

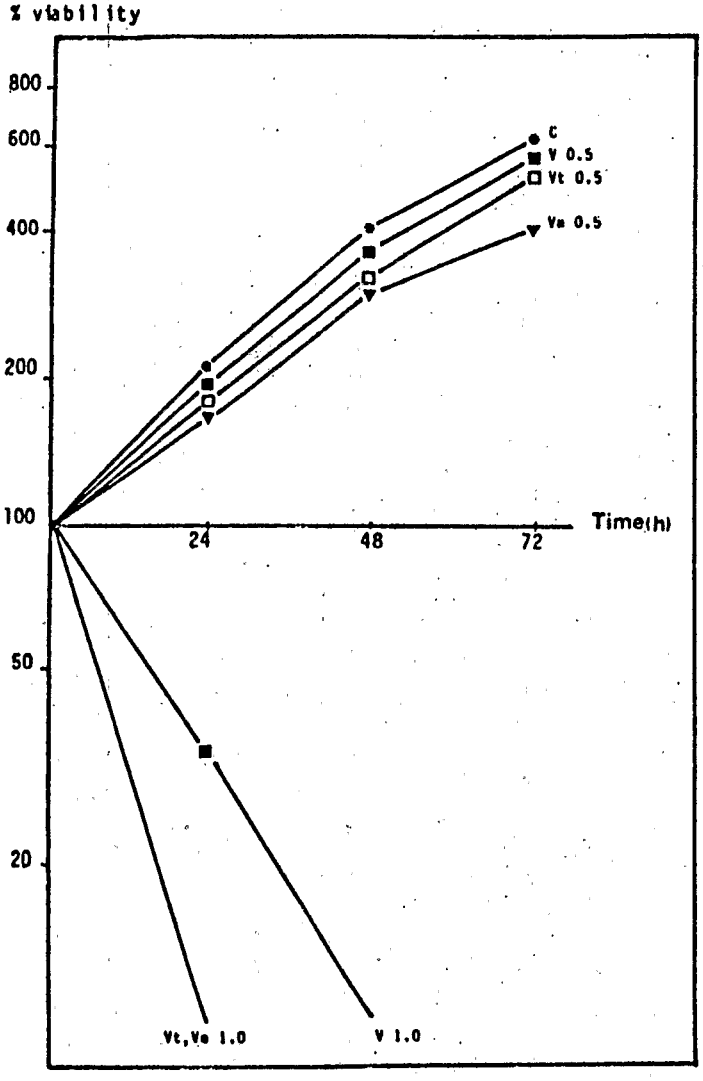


Fig. 2. In vitro dose-dependent cytotoxicity of vatine (V), vatamine (Vt) and vatamidine (Vm) on HTC cells (concentrations in μM).

concentration, exhibit similar activity except for the isopsychotridines, for which at the end of the incubation period of 72 h, isopsychotridine E is more toxic than isopsychotridine D. In this case, stereomerism could have a slight effect on the cytotoxic activity of these alkaloids.

Effect of the mode of polymerisation

Calycanthine (cal), a dimeric tetrahydroquinoline alkaloid that often occurs

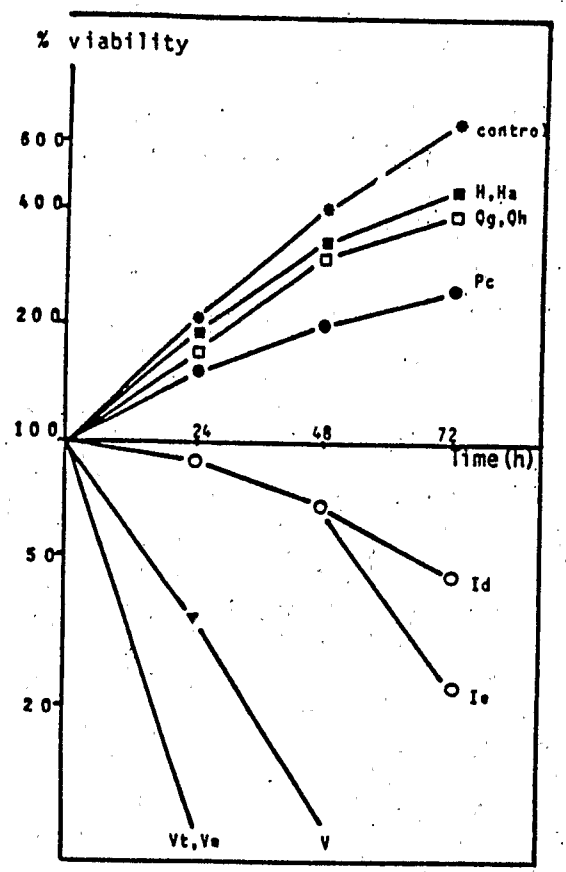


Fig. 3. Influence of the number of tryptamine units on the cytotoxic effects of polyindolenine alkaloids on HTC cells in vitro.

with pyrrolidinoindolinic alkaloids (Gorman et al., 1971; Hall et al., 1967) was used for this assay. It is closely related to these compounds by its biosynthetic pathway and derives from the oligomer chimonanthine consisting of two methyltryptamine units linked by a $\beta-\beta'$ bond (Hendrickson et al., 1964). In calycanthine, a particular type of bond occurs (Hall et al., 1967). Chimonanthine, its corresponding pyrrolidinoindolinic dimer, was not available, therefore, we chose hodgkinsine to compare the influence of the two different types of linkages: the two alkaloids were tested at concentrations of 2.5×10^{-6}, 4×10^{-6} and 8×10^{-6} M. The viability curves show that calycanthine is inactive at the three different concentrations while hodgkinsine exhibits an inactive to strong cytotoxic effect according to the dose.

The mode of polycondensation of the Nb-methyltryptamine units through the two types of bonds $C_{3a}-C_7$ and $C_{3a}-C_{3a'}$ usually encountered in polyindo-

lenine alkaloids seems to be necessary for their cytotoxic effects. The inactivity of calycanthine could also be due to steric effects.

Comparison with some well known antitumor agents

Antitumor agents can be classified into four main groups according to their mean of action; alkylators, antimetabolites, antimitotics and intercalators. Some members of these groups were chosen for comparison with pyrrolidinoindolinic alkaloids. The cytotoxic properties of each of the antitumor agents was previously studied on HTC cells and revealed that there were no dose-effect relationships at the dose levels of 10 and 100 μM with the different chemotherapeutics agents used, except for the intercalators, which show a dose-dependent effect within certain limits (Bounthanh, 1985). Hodgkinsine (the least active) and vatine were chosen for this study. The viability curves are presented in Fig. 4 and show that colchicine, a tubuline inhibitor, had a mitostatic effect during the 72 h of the experiment; methotrexate (Mx), an antifolate, at 10 μM inhibits cell multiplication during 48 h and then becomes

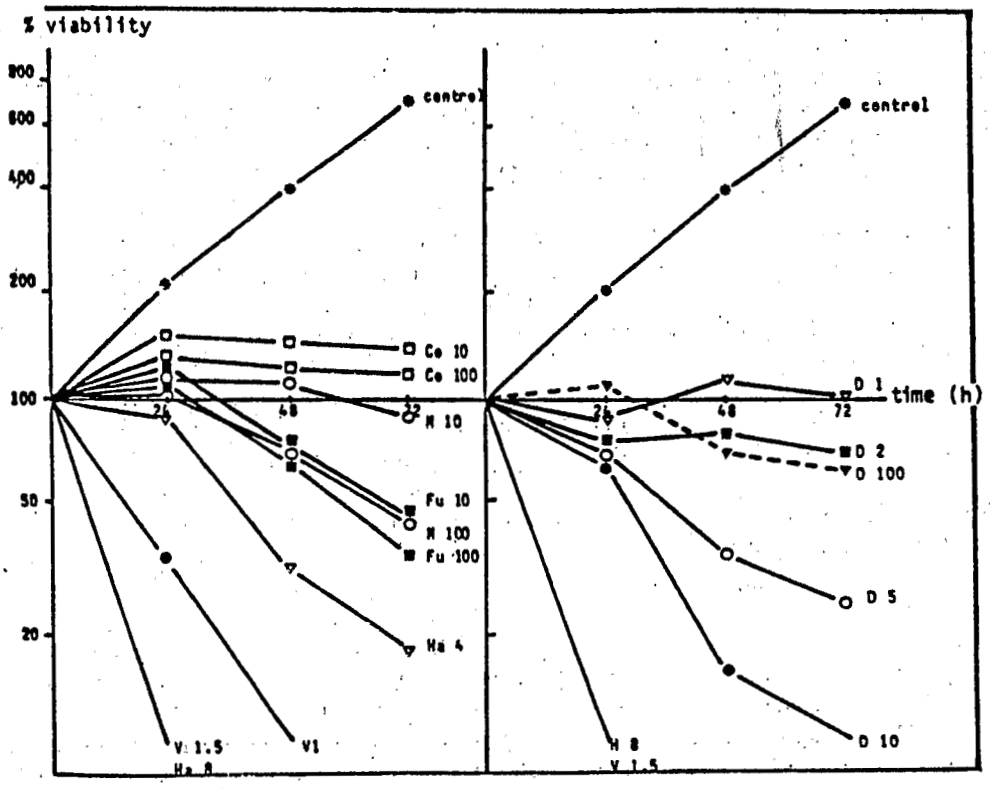


Fig. 4. Activity of some antitumor agents on HTC cells in vitro (concentrations in μM). Co, colchicine; Fu, 5-fluorouracil; M, methotrexate; D, doxorubicine; V, vatine; Ha, hodgkinsine A.

slightly toxic at the end of 72 h of contact; 5-fluoro uracil, an antimetabolite, at 10 and 100 μM as well as Mx at 100 μM produce 60 to 70% mortality after 72 h of incubation time. Doxorubicine (intercalator) showed dose-effect relationship: at 1 μM; it is inactive to slightly cytotoxic but its activity increases with increasing the dose up to 10 μM where 100% cell mortality occurs after 72 h. Surprisingly, increasing the dose to 100 μM results in decreasing its activity.

Hodgkinsine A at 8 μM and vatine in a much lower concentration of 1.5 μM are potent cytotoxic agent with 100% cellular mortality after 24 h. During the same period, no cellular mortality was obtained with any of the antitumor agents used.

The cytotoxicity to HTC cells of the antitumor agents used in this study is lower than that obtained with polyindoline alkaloids.

Conclusion

Polyindoline alkaloids are strong cytotoxic agents in micromolar range to HTC cells in vitro. Their activity is dose-dependent and is a function of the time of contact. Moreover, increasing the molecular weight (i.e. number of units) increases the activity. The position of the $\beta-\beta'$ bond in these substances is also important for activity. Thus the molecules with high molecular weight and/or with a terminal single tryptamine unit are the most toxic. The toxicity of these compounds compared to some antitumor agents is in favour of a complex mechanism of action.

Further studies on various other cell lines, i.e. human tumoral ($Molt_4$) and normal cell lines, presently in progress, support the results and should allow a better comprehension of the cytoinhibitory properties of these peculiar alkaloids.

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