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An evaluation of the suitability of the onion test for studying the biological activity of potential antitumor drugs on the example of ledakrin

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Abstract

The onion test was employed in this study. The effect of ledakrin on the synthesis of DNA and protein in meristematic cells of onion adventitious roots was examined. Comparison of the results revealed their similarity to data on animals and bacteria. It was found that inhibition of DNA synthesis is high and seems to precede the inhibition of protein synthesis (which is very low). Also, the difference between the lowest active concentration and lethal dose is, similarly as in mammals, slight (low therapeutic index). It was found that ledakrin is quickly taken up from the incubation medium by the roots; during the first six hours of incubation, a major part of the supplied compound enters into them. During postincubation, the compound is excreted into the medium by the roots; during the first day of postincubation, 10%, during the following days, 0.2–2.0% of the amount taken up by the roots is excreted. During the first hour of incubation, the drug enters all of the root meristematic tissues, while after 7 days of postincubation, this compound, or its metabolites, is still present in small amounts in the meristem.

Key words: *anion test, ledakrin uptake, protein synthesis, DNA synthesis*

INTRODUCTION

Initial tests of the biological activity of potential antitumor drugs are conducted mainly on mammals and mammalian cells and on bacteria (Konopa et al. 1969, Chotkowska and Konopa 1972, Radzikowski

1974, Pawlak et al. 1983). The use of plant tests to this end presents the question of the usefulness of the results in further testing of the compound, due to the differences in the structure of plant and animal cells and organisms (Grant 1978, Nilan 1978). The suitability of plant tests for screening and initial studies of the biological activity of antitumor drugs will thereby be determined by the similarities of the drug's effect on human or animal cells and on plant cells.

The objective of this paper was to determine the suitability of the onion test for such studies, using ledakrin (1-nitro-9(3-dimethylaminopropylamino)-acridine, a Polish anticancer drug (Radzikowski 1974). This problem was touched on in the paper by Antosiewicz (1984) where it was shown that ledakrin binds covalently with the DNA of onion meristematic cells, similarly as in humans, mammals and bacteria. This binding is considered to be the main pathway of the drug's cytostatic and antitumor action (Gniazdowski et al. 1979, Krawczyk 1982, Konopa et al. 1983), and its presence in meristematic cells indicates that ledakrin is similarly metabolized as in mammalian and bacterial cells (Antosiewicz 1984). In this study, the effect of ledakrin on other important processes, DNA and protein biosynthesis, was studied. These processes have already been studied in humans, animals and bacteria (Chotkowska and Konopa 1973, Gniazdowski et al. 1979), thanks to which it is possible to compare the effect of ledakrin on the cells of these organisms and on plant cells.

In this study, an initial evaluation of the conditions for conducting experiments using the onion test and ledakrin has been made, determining the uptake of the drug by roots and its presence in meristematic cells.

MATERIALS AND METHODS

Onion (*Allium cepa* L.) roots were cultured as previously described (Antosiewicz 1984, Wierzbicka 1987a, Wierzbicka and Antosiewicz 1988). In the cases in which postincubation lasted longer than 3 days, Hoagland's medium (Hoagland and Arnon 1938), diluted 1:8 and suppplemented with 0.1 cm^3 A-Z microelements per 1 dm^3 undiluted medium, was used. Solutions of ledakrin in deionized water of the following concentrations were used: 0.05 ppm (causing the first noticeable changes), 0.1 ppm (sublethal) and 0.125 ppm (lethal after 24 hrs incubation); incubation times: 10 min, 1m 3, 6, 12 and 24 hrs, postincubation: I, II, III, V, VII, and IX days.

LEDAKRIN UPTAKE

($1\text{-}^{14}C$)-ledakrin with a specific activity of 9.0 mCi $mmole^{-1}$ was used in making up the incubation mediums.

Ledakrin uptake was determined on the basis of the decrease of the radioactivity of the incubation mediums. The radioactivity of the postincubation mediums was also measured. To a 0.5 cm^3 sample, 5 cm^3 of Bray's (Bray 1960) scintillator were added and counted for 10 min in a Beckman LS-100 C liquid scientillation counter. Radioactivity measurements were made for liquids from 10 culture vessels per combination (three samples from each vessel were counted).

The rate of penetration of ledakrin into the root meristem and duration of its presence there were evaluated autoradiographically. For this purpose, roots 1 cm in length were fixed in formacetoalcohol (1:1:18) for 24 hrs, embedded in paraffin and then sectioned longitudinally into 6 μm thick sections. Hydrated sectioned samples were used to make autoradiograms on Kodak AR-10 emulsion. Exposition was for 60 days at +4°C. Post-exposition staining was done using 0.25% toluidine blue in 1% borax, after which the emulsion was destained with 0.1 N HCl. The samples were dehydrated in isopropyl alcohol. A comparative, approximate evaluation of the amount of ^{14}C-ledakrin taken up was done on three sections from each experimental combination.

DETERMINATION OF THE LEVEL OF DNA AND PROTEIN SYNTHESIS

Onion roots were exposed to (^3H-methyl)-thymidine 3 μCi (spec. act. 28 Ci $mmole^{-1}$ TRK 120, Batch 120, Amersham) and to (4.5-^3H)leucine 8 μCi (spec. act. 40 Ci $mmole^{-1}$, TRK 170, Batch 66, Amersham) for the final 2 hours of incubation or postincubation, appropriately.

All of the autoradiograms were made using aceto-orcein squashed slides of apical meristems of roots fixed for 3 hrs in acetoalcohol (3:1). Kodak AR-10 emulsion was used, exposition was for 14 days at +4°C.

The following calculations were made for 5 slides from each combination:

1. After exposure to ^3H-thymidine, the number of grains over 60 nuclei and the number of labeled cells per 1000 interphase cells were determined on a slide made from one apical meristem.

2. After exposure to ^3H-leucine, the number of grains over the entire cell per 1000 interphase cells were counted.

The results were analysed using Student's t-test (Greń 1968) which indicates which of the experimental results differ to a statistically significant degree from control values.

RESULTS

LEDAKRIN UPTAKE

Changes in the relative radioactivity of the incubation mediums are shown on Fig. 1. This expresses the amount and rate of uptake of

^{14}C-ledakrin by roots. After 6 hrs of incubation with ledakrin at 0.1 and 0.05 ppm, its content in the vessels equalized and amounted to 287 and 239 cpm (the difference is not statistically significant). The amount of the compound taken up during this time was about 3 times greater from the 0.1 ppm medium (about 950 cpm) than from the 0.05 ppm medium (about 331 cpm), thereby the uptake rate at this time was disproportionately higher for the roots submerged in the ledakrin solution of the higher concentration. The roots which had taken up more ledakrin,

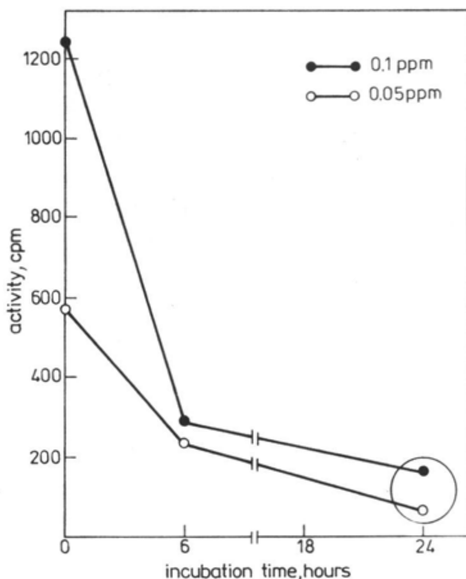

Fig. 1. Changes in the relative radioactivities of incubation mediums during incubation with ^{14}C-ledakrin. Differences between the circles and control values are statistically significant

continued to do so (those in the 0.1 ppm solution) more rapidly than those which had taken up less, since otherwise, the observed equalization of concentrations would not have taken place.

The uptake of the remaining, small amount of ledakrin during the subsequent 18 hrs of incubation was slower in the roots which had taken up the larger amount of the compound. Finally, then, after 24 hrs of incubation, the onions exposed to a two times higher concentration of ledakrin (0.1 ppm as opposed to 0.05 pm) took up twice as much of the drug, which in both cases, amounted to 87% of the initial amount of the drug. When comparing the amount of ledakrin taken up by roots placed in mediums containing the studied concentrations of the drug, in both cases it is seen that the major part of the drug was taken

up during the first 6 hrs of incubation — for the 0.1 ppm concentration it was 77% of the supplied amount (and 88% of the entire amount taken up during 24 hrs of incubation), for 0.05 ppm — 58% and 66%, respectively.

Examination of the activity of the post-incubation solutions revealed that ledakrin was excreted by the roots (Fig. 2), in spite of the fact

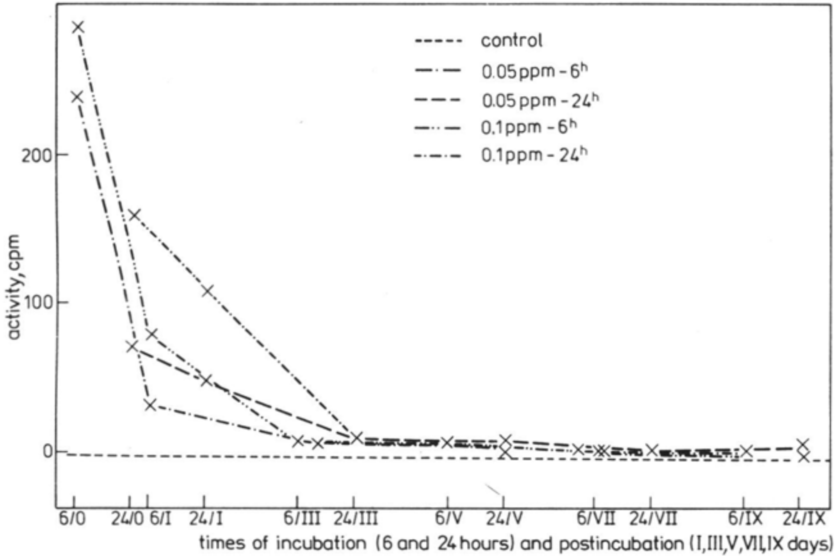

Fig. 2. Changes in the relative radioactivities (cpm) of incubation and postincubation medium after application of ^{14}C-ledakrin. Initial radioactivity of incubation mediums: 0.05 ppm — 570 cpm; 0.1 ppm — 1237 cpm

that they were washed very intensely before being transferred from the incubation medium to the postincubation water. During the first day of postincubation, the excreted amount equaled about 10% of the amount taken up, during the following days, only trace amounts were excreted, amounting to 0.2–2.0% of the amount taken up by the roots. It is not known, however, if the radioactivity found in the postincubation medium was bound with the initial form of the drug or with its metabolites. In addition, the ledakrin removed from the roots during washing was not taken into account. This washing was, however, indispensable due to the removal of the unbound ledakrin from the roots.

The results of the estimation of the ledakrin content in the individual primary meristems of the root apical meristem during the experiment after various times of incubation and postincubation, are presented in Table 1. After only 10 min, very small amounts of the drug were observed in the root cap and protoderm. Singular traces were visible in the ground

Table 1

Relative amounts of ledakrin in the meristem after exposition to 0.1 ppm ^{14}C-ledakrin

Time incubation and postincubation	Ledakrin content			
	root cap	protoderm	ground meristem	protocambium
10 min	+*	singular traces	singular traces	–
1 hr	++	++	+	singular traces
3 hrs	+++	++	++	+
6 hrs	++++	++++	++++	+++
6/I	++++	++++	++++	++++
6/II	+++	+++	+++	+++
6/VII	+	+	+	+
24 hrs	+++++	+++++	++++	++++
24/I	+++++	++++	++++	++++
24/II	++++	++++	++++	++++
24/III	+++	+++	+++	+++
24/VII	+	+	+	+

Incubation time (hrs): 1, 3, 6, 24 and 10 min. Postincubation time (days): I, II, III and VII.
* The number of plusses denotes the increasing degree of darkening of the photographic emulsion over the meristem.

meristem although they were lacking in the protocambium. After 1 hr, the content of the drug in the tissues increased, its presence was visible in the protocambium, albeit in the least amount. During continued incubation, the amount of ledakrin increased in all of the apical meristem tissues, although its content after 24 hrs of incubation was only slightly greater than after 6 hrs. There was generally less ledakrin in the protocambium than in the remaining tissues, although this difference was very slight.

On the first day of postincubation, the amount of ledakrin was the same as after 6 or 24 hrs of incubation. A distinct decrease the ledakrin content of the meristem was observed during the third day of postincubation, while after 7 days it was still present in small amounts in meristematic cells.

CHANGES IN DNA AND PROTEIN SYNTHESIS AFTER ADMINISTRATION OF LEDAKRIN

Ledakrin strongly inhibited DNA synthesis both during incubation and postincubation (Fig. 3). A simple relationship was found between the degree of inhibition of DNA synthesis and the concentration and duration of ledakrin action. It was clearly evident that the drug's effect on DNA synthesis extended from incubation to postincubation. Along with rising ledakrin concentrations and time of action, inhibition of DNA synthesis first took place during postincubation (0.05 ppm — 24/I, 24/II), and only at higher concentrations, during incubation. The lethal ledakrin concentration

caused a complete halt in DNA synthesis during postincubation. The reduced level of DNA synthesis during incubation and postincubation slowly increased during postincubation after previous incubation with ledakrin at non-lethal concentrations.

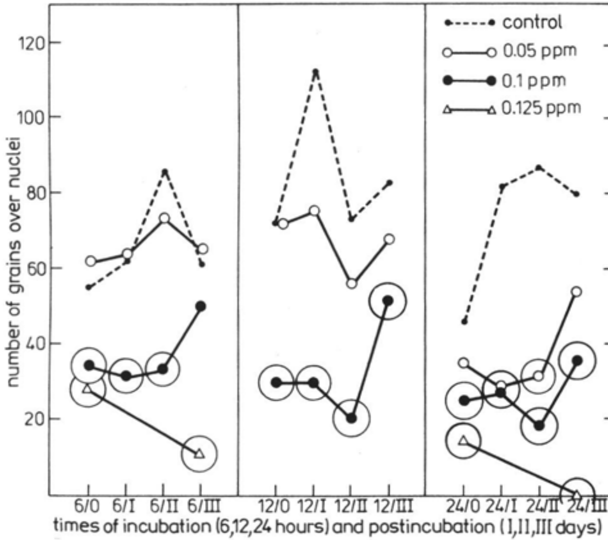

Fig. 3. The effect of ledakrin on the incorporation of ^{3}H-thymidine by cell nuclei of root apical meristem cells. Differences between circles and control values are statistically significant

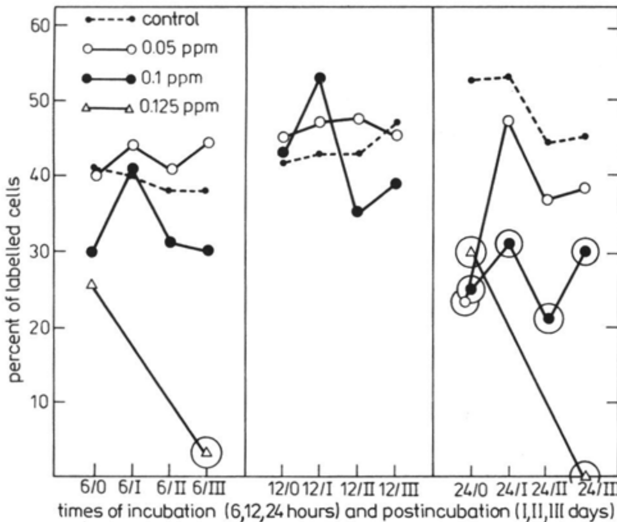

Fig. 4. The effect of ledakrin on the number of labeled meristematic cells after application of ^{3}H-thymidine. Differences between circles and control values are statistically significant

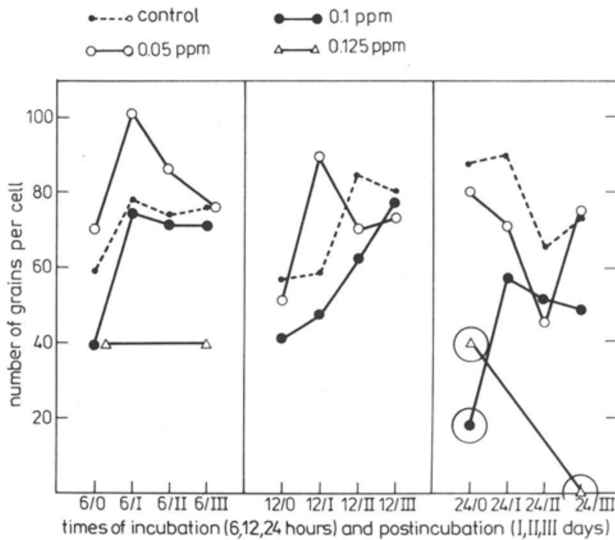

Fig. 5. The effect of ledakrin on the incorporation of ^{3}H-thymidine by meristematic cells. Differences between circles and control values are statistically significant

The decrease in the number of cells synthesizing DNA (Fig. 4) was less distinct than the decrease in DNA synthesis in the nucleus. A statistically significant drop was visible only after 24 hrs of incubation with ledakrin at all three studied concentrations, and the number of cells synthesizing DNA returned to the control level during postincubation only when the concentration of 0.05 ppm was used. The highest used ledakrin concentration caused a fall in the number of cells synthesizing DNA to zero after the time 24/III.

The effect of ledakrin on protein synthesis was significantly weaker than its effect on DNA synthesis (Fig. 5). A statistically significant fall in the level of protein synthesis was apparent only after 24 hrs of incubation at 0.1 ppm. The lethal concentration — 0.125 ppm — caused a gradual fall of the level of protein synthesis to zero during postincubation.

DISCUSSION

Higher plant test systems are gradually being recognized as good indicators of cytotoxicity and of cytogenetic and mutagenic effects of chemical compounds which may pose a danger to human genetics (Grant 1978, Fiskesjö 1982). Hence, in routine testing for mutagens, they are becoming an attractive alternative to tests run on mammals and other organisms (Grant et al. 1981). However, the applicability of plant tests

to initial testing of the biological activity of potential antitumor drugs remains an open question. The main problem is the physiological and phylogenetic differences among plant, animal and human cells. Connected with this is the unknown metabolism of the drug in the cells, which is of basic importance in the manifestation of its antitumor properties. These reservations also pertain to the onion test used in this study, in spite of the fact that it is widely applied to study the biological activity of a wide variety of factors, including antitumor preparations (Grant 1982). However, attempts are being undertaken more often to fill in the gaps. Spano and Takahashi (1981) showed, studying the drug Nifurtimax, that *Drosophilla melanogaster*, rat bone marrow cells and *A. cepa* apical meristem cells are sensitive indicators for uncovering the mutagenic and klastogenic properties of drugs. When comparing the activity of antitumor drugs in the onion test with that in animal tests. Amman and Safferman (1958) demonstrated a positive correlation for actinomycin D and azaserine.

Comparative studies using compounds other than drugs have demonstrated, among others, their similar activity in the onion and fag tests and in mammalian cell cultures, as well as a strong correlation between the occurrence of chromosome anomalies and mutagenic activity (Grant 1978, Fiskesjö 1982). The value of plant tests is being enhanced by the still few, in comparison with animal material, reports on the occurrence of various metabolic transformations of exogenous compounds in a way similar as in animals.

The results presented in this study on the effect of ledakrin on the synthesis of DNA and protein in meristematic cells have shown the similarity of the studies processes in onion and in animals and bacteria. Konopa et al. (1976) have shown by studying the biosynthesis of DNA, RNA and protein in HeLa cells after 6 hrs of incubation with ledakrin, that the incorporation of radioactive precursors into DNA and RNA is inhibited very strongly, while incorporation into protein is affected less. A lower concentration of ledakrin inhibited DNA and RNA synthesis by 40–50%, while protein synthesis remained unaffected. Only a higher concentration of ledakrin lowered the biosynthesis of both nucleic acids and proteins. According to Chotkowska and Konopa (1973), in *Euglena gracilis*, under the influence of ledakrin, after the first division, protein biosynthesis is not inhibited, although DNA and RNA synthesis are. Only after the second division are DNA, RNA and protein synthesis all inhibited. The inhibition of the incorporation of ^{3}H-uridine into KB cells from human pharynx cancer, was greater after 30 min of incubation than of ^{14}C-phenylalanine incorporation, which indicates that the inhibition of RNA synthesis was the primary event (Filipski et al. 1975). The N-oxide derivative of ledakrin, C-684, also strongly inhibits DNA synthesis while not affecting protein synthesis (Pawlak 1982). Therefore, the conclusion

that inhibition of DNA synthesis precedes inhibition of protein synthesis is true for meristematic tissues also.

The lowering of the template activity of DNA as the result of binding ledakrin to DNA has been demonstrated in the process of transcription (Gniazdowski et al. 1975, Krawczyk 1982). It results from this that the inhibition of nucleic acid and protein synthesis, due mainly to the formation of bonds between ledakrin and DNA (Konopa et al. 1976), may also take place in onion meristematic cells, since the covalent linkage of ledakrin and meristem DNA has been demonstrated (Antosiewicz 1984).

It should, however, be added that in spite of the fact that binding of ledakrin with DNA is considered to be the basic phenomenon responsible for the biological activity of the drug, ledakrin can also bind in vivo with other cell macromolecules — RNA and protein (Gniazdowski et al. 1979, Pawlak et al. 1983). The metabolism of ledakrin can lead to the formation of new derivatives, able to bind with proteins, and which are different from those metabolites which bind with DNA and are responsible for the cytotoxic effect of the drug (Wilson et al. 1984).

The similarity of the effect of ledakrin on living plant cells and on animal and human cells also pertains to the size and range of active doses of the drug. The difference between the lowest active dose and lethal dose is in the onion, similarly as in mammals, very small — it has a low therapeutic index (Kwaśniewska-Rokicińska and Winkler 1969), and the active doses of the drug used in the onion test are close to those used in other tests (Antosiewicz 1985, 1988).

Due to the similarity of the effect of ledakrin on meristematic onion cells and on human or animal cells as well as on microorganisms, the onion test can be considered promising in the screening for and initial testing of potential antitumor drugs. However, further studies are still necessary in order to understand the metabolism which various cytostatics undergo in different plant tests, as well as the basic mechanism of their action in comparison with human and animal material.

The basic experiments conducted during this study dealt with determining the rate of ledakrin uptake by roots, and its presence in the meristem during the experiment indicated that in the studied material, a major part of the drug which had been taken up during 24 hrs of incubation, entered into the root tissues during the first 6 hrs. After only 1 hr, slight amounts of the drug were present in all of the meristem's tissues. The disproportionately more rapid uptake of ledakrin during the first hours of incubation at the higher concentration may cause a stronger reaction than expected for this concentration. The quick depletion of the drug from the incubation medium is the reason that, in the case of a lengthy incubation, it only really lasts a few hours, and the observed effects are due to the metabolism of the drug within the root. A similar

effect was observed while subjecting onion roots to lead salts (Wierzbicka 1984). During postincubation of roots in water, a certain amount of leadakrin was secreted from the roots, making postincubation initially very weak incubation. The ledakrin supplied to the roots was present in the meristem even after 7 days of postincubation. It is known that ledakrin undergoes metabolic processes in the onion root meristematic cells which produce a derivative capable of forming covalent linages with DNA (Antosiewicz 1984, 1987b). Since it has been demonstrated that in animal cells, at least 6 different metabolites of ledakrin arise (Pawlak and Konopa 1979), and nothing is known about the differences in their activity, it cannot be determined if the label observed in the meristem is from the initial form of ledakrin, or from one of its metabolites.

It is also remarkable how, in spite of the intense growth of the roots, and after so many days after the drug had been given, it was still present in the meristem. The control roots grew approx. 10 cm, at 0.1 ppm ledakrin, about 6 cm (Antosiewicz 1985). This presence may result from the metabolism of ledakrin in the roots. Known, for example, is the phenomenon of translocation of absorbed substances from the higher parts of the root in the apical direction, through free water spaces, and the movement of substances downwards does not necessarily need to be connected only with any specific needs of the meristematic tissue (Sytnik et al. 1977, Wierzbicka 1984, 1987c).

The results obtained in this study and in a previous paper (Antosiewicz 1984), point to the usefulness of plant tests in the testing of cytostatics. The basic information gathered on the uptake of the drug and its presence in the meristem may be of value in the interpretation of the results of further studies on the drug.

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Ocena przydatności testu Allium do badania aktywności biologicznej preparatów potencjalnie antynowotworowych

Streszczenie

W pracy zastosowano test *Allium*. Zbadano wpływ ledakrinu na syntezę DNA i białek komórek merystematycznych korzeni przybyszowych cebuli. Porównanie uzyskanych wyników wykazało ich podobieństwo do danych dla zwierząt i bakterii. Stwierdzono, że hamowanie syntezy DNA jest silne i wydaje się być zdarzeniem pierwotnym w stosunku do hamowania syntezy białek (która jest bardzo słaba). Także różnica między najniższym stężeniem aktywnym i letalnym jest, podobnie jak u ssaków, niewielka (mały indeks terapeutyczny).

Ustalono, że ledakrin jest szybko pobierany przez korzenie z płynów inkubacyjnych; podczas pierwszych 6 godzin inkubacji dostaje się do nich większa część podanego związku. Preparat w postinkubacji jest wydalany z korzeni na zewnątrz do wody, przy czym w pierwszej dobie postinkubacji jest to 10‰ a w następnych — 0.2-2% ilości pobranej przez korzenie. Lek w ciągu pierwszej godziny inkubacji dostaje się do wszystkich tkanek merystematycznych korzenia, a po siedmiu dniach postinkubacji związek ten, bądź jego metabolity obecne są jeszcze w małych ilościach na terenie merystemu.