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IN VITRO BINDING OF METABOLICALLY ACTIVATED [^{14}C]-LEDAKRIN, OR 1-NITRO-9-^{14}C-(3′-DIMETHYLAMINO-*N*-PROPYLAMINO) ACRIDINE, A NEW ANTITUMOR AND DNA CROSS-LINKING AGENT, TO MACROMOLECULES OF SUBCELLULAR FRACTIONS ISOLATED FROM RAT LIVER AND HeLa CELLS*

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Abstract—A new antitumor drug named Ledakrin or Nitracrine, 1-nitro-9-(3′-dimethylamino-*n*-propylamino)acridine, which has been shown to be a latent DNA cross-linking agent in both mammalian and bacterial cells, was investigated to determine whether it irreversibly binds to cellular macromolecules *in vitro*. Incubation of [^{14}C]-Ledakrin with subcellular fractions of either rat liver or HeLa cells in the presence of a NADPH-regenerating system led to an irreversible binding of as much as about 30 per cent of the drug radioactivity (up to 57 nmoles/mg protein) with subcellular macromolecules after exhaustive extraction with cold trichloroacetic acid, ethanol and ether. The binding seems to be covalent. The difference between irreversible binding in the presence of intact and heat-inactivated enzymatic subcellular fractions indicates that the metabolites of the drug, rather than Ledakrin itself, are responsible for the irreversible binding with macromolecules *in vitro*. The dependence of the macromolecule binding of Ledakrin radioactivity with subcellular macromolecules of post-mitochondrial or microsomes on microsomal enzyme, on substrate concentration, oxygen and NADPH, as well as induction of this reaction with phenobarbital or 3-methylcholanthrene rat pretreatment, indicates that the oxidative macromolecule binding of Ledakrin metabolites is catalyzed *in vitro* by mixed-function oxidases, probably by the unspecific drug metabolizing system involving cytochrome P-450 of liver microsomes. Irreversible binding *in vitro* was less pronounced under anaerobic conditions than in incubations under air. The reductive irreversible macromolecule binding of Ledakrin metabolites is catalyzed *in vitro* by unknown rat liver enzymes resistant to allopurinol or dicoumarol inhibition. To account for oxidative binding of Ledakrin through a metabolic activation *in vitro*, three pathways are considered likely: (1) C-hydroxylation; (2) *N*-alkylhydroxylamine formation and (3) aromatic *N*-hydroxylation. The elevated oxidative macromolecule binding of Ledakrin metabolites when an epoxide hydrase was inhibited is evidence for the formation of a reactive acridine epoxide intermediate during the drug binding reaction. The ineffectiveness of SKF 525-A, a specific inhibitor of microsomal C-oxidation, in decreasing the irreversible binding is indirect evidence that besides microsomal C-oxidation other oxidative activations are involved. Arylhydroxylamines formed under air *in vitro* with all subcellular fractions studied, as determined colorimetrically. An aliphatic *N*-hydroxylation of the amino group of Ledakrin side chain can be involved in the drug oxidative binding *in vitro* too. To account for irreversible macromolecule binding of Ledakrin metabolites under highly anaerobic conditions, a *N*-arylhydroxylamine arising from the nitro group reduction seems to be an intermediate determined colorimetrically. Moreover, the metabolism of the nitro group of Ledakrin to its parent 1-*N*-hydroxylamine was directly related to the irreversible binding of the drug metabolite(s) with subcellular macromolecules *in vitro* under nitrogen. Reduced glutathione trapped *in vitro* reactive electrophilic Ledakrin metabolite(s) formed most probably by establishing a chemically stable thioether bond and thereby protected macromolecules against irreversible binding. Finally, four reactive species are postulated in the irreversible macromolecule binding of Ledakrin metabolites *in vitro*.

INTRODUCTION

The interaction of nitroacridines with cellular macromolecules *in vivo* and *in vitro* has been investigated to elucidate the mode of action of 1-nitro-9-(3′-dimethylamino-*n*-propylamino)acridine, an antitumor drug named Ledakrin (WHO recommended name Nitracrine, previously referred to as the compound C-283) [1–3]. In cell systems, 1-nitroacridines exhibited great and comparable cytotoxic activity, while the derivatives with the nitro group in another position of acridine nucleus were inactive [4–6]. Attempts to find a similar relationship between the position of the nitro group and activity in cell-free systems have been so far unsuccessful since all nitroacridines formed *in vitro* complexes with DNA with approximate stoichiometry and apparent binding constants which led to similar stability of secondary structure and matrix activity of DNA in these

* The results were presented at the Meeting of the Mode of Action of Ledakrin, Technical University, Gdańsk, Poland, 7–8 June, 1977 (Abstr.) and at 12th FEBS Meeting, Dresden, East Germany, 2–8 July, 1978 (Abstr.).

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complexes [6–12]. We have shown that the most cytotoxic agent, Ledakrin, interacted covalently *in vivo* with cellular macromolecules, which included DNA, of Ehrlich ascites tumor cells [10, 12, 13, 15], HeLa cells grown in tissue culture [12–14] and bacteria [13, 14]. The cross-linking of mammalian DNA by Ledakrin *in vivo* has been also confirmed for L 1210 cells [74]. Furthermore, we evidenced that Ledakrin as well as other 1-nitroacridines cross-linked DNA of both mammalian [12, 14–16] and bacterial [14, 16] cells *in vivo*. The cross-linking of cellular DNA of sensitive cells by Ledakrin or other 1-nitroacridines is very likely to be the primary event responsible for their cytotoxic activities, against both bacterial and mammalian cells *in vivo* [16]. The metabolic activation *in vivo* resulting in the formation of at least one or two binding sites in the acridine molecule seems to be an essential prerequisite for the mono- or bifunctional covalent binding of 1-nitroacridines with cellular DNA [12, 14, 16]. Therefore, we wondered whether a covalent binding of the drug with cellular macromolecules of tissue homogenates *in vitro* might help us to understand the mechanism of covalent macromolecule binding of Ledakrin and what are the metabolic activations which cause such a binding. A number of experimental studies have provided evidence that some indirect-acting carcinogens or drugs are metabolized to their activated intermediates by different liver enzymes [17–20]. Hence, we investigated an irreversible binding of activated Ledakrin with rat liver subcellular macromolecules and albumin with *in vitro* experiments, and covalent binding of the drug with subcellular macromolecules *in vitro* has been used as a measure of the amount of active metabolite(s) formed [17–19]. We examined also samples from HeLa cells grown in tissue culture to find out whether these drug-sensitive cells [6, 12–14, 16] behave *in vitro* similarly. In this report we would however prefer the term 'irreversible binding' instead of 'covalent binding' for a macromolecule binding which can not be split by exhaustive solvent extraction, because of the lack of a definite evidence for the covalent binding of the drug with macromolecule(s).

MATERIALS AND METHODS

Labeled compounds and other reagents

[^{14}C]-Ledakrin [1-nitro-9-^{14}C-(3′-dimethylamino-*n*-propylamino)acridine hydrochloride, WHO recommended name Nitracrine, specific activity 7.36 mCi/mmole] [21] and nonlabeled Ledakrin [1, 2] were provided by Prof. Dr Andrzej Ledóchowski, Dept. of Pharmaceutical Technology and Biochemistry, Technical University of Gdańsk, Poland. The chemical purity of both compounds was greater than 95 per cent [14]. NADP, NADPH, glucose-6-phosphate dehydrogenase, bovine serum albumin, 3-methylcholanthrene (3-MC), allopurinol, dicoumarol, dithiotreitol, *N*-acetyl-L-alaninegliclylglycine ethyl ester HCl, and *N*-acetyl-tyrosine, were all purchased from Sigma, USA. Glucose-6-phosphate and cyclohexene oxide from Koch-Light, England; nicotinamide from Loba, Austria; and reduced glutathione from BDH, England were obtained. Sodium phenobarbital was a gift from United Pharmaceutical Industries Polfa, Poland; SKF 525-A [2-diethylaminoethyl-2, 2-diphenylvalerate·HCl] was kindly donated by Smith, Kline and French, England. All other reagents either were of analytical reagent grade or were distilled prior to use.

Animals

Male Wistar albino rats (Dept. of Pathology, Medical Academy, Gdańsk, Poland) weighing 180–240 g were used and maintained on a commercial diet and water ad lib. Rats were divided into three subgroups. The first was a control subgroup. The second subgroup received i.p. sodium phenobarbital in 0.9% NaCl solution at a dose of 80 mg/kg of body weight, once daily, for 3 consecutive days. The third subgroup received i.p. 3-methylcholanthrene in corn oil at a dose of 25 mg/kg of body weight, once daily, for 3 consecutive days. The animals were fasted for the last 18 hr and killed 24 hr after the last injection. The control animals received injections of the appropriate vehicle.

Tissue culture

HeLa cells were grown in monolayer culture in Minimal Essential Eagle's medium (Hank's balanced solution) supplemented with 10% calf serum, penicillin and streptomycin. Cells (5×10^6) were plated in 350 ml Roux flasks containing 25 ml of the medium. One day after seeding the medium was replaced with a fresh one. After 24 hr the medium was removed, the cells were trypsinized and suspended in ice-cold sucrose-phosphate (0.25 M sucrose, 0.05 M sodium phosphate buffer of pH 7.4 containing 1.15% KCl). HeLa cells were harvested by centrifugation at 4° at 750*g* for 15 min, and washed once with ice-cold sucrose-phosphate. After being again collected by centrifugation the cells were suspended in 3 vol. of ice-cold sucrose-phosphate.

Preparation of tissue homogenates and subcellular fractions

Control animals were killed by cervical dislocation, exsanguinated, and then all subsequent steps were performed at 0–4° using ice-cold solutions and glassware. Livers were immediately removed, weighed, minced, and washed as free from hemoglobin as possible with sucrose-phosphate. Liver samples were homogenized with a motor-driven glass-Teflon homogenizer (Potter-Elvehjem type) in 3 vol. of sucrose-phosphate. The homogenate was centrifuged at 750*g* for 15 min, the pellet was discarded and the supernatant again centrifuged as above. The supernatant was centrifuged at 10,000g (Beckman J-21B Centrifuge, JS-7.5 rotor) for 30 min, the pellet discarded, and the supernatant recentrifuged at 10,000*g* for 30 min. The supernatant fraction was decanted; the part of this supernatant was used as post-mitochondrial supernatant (10,000*g* supernatant) whereas the rest of the supernatant served subsequently as the source of microsomes and cytosol. The latter supernatant fraction was centrifuged at 105,000*g* for 60 min in a Beckman L5-50 ultracentrifuge with Ty-50 rotor. The crude cytosol was separated from the crude microsomal pellets by careful decantation. The crude microsomal pellets were suspended in sucrose-phosphate, then these washed

microsomes and the crude cytosol were both separately recentrifuged at 105,000*g* for 30 min. The supernatant fluid from centrifuged crude cytosol was used as cytosol fraction (cytosol). The pellet obtained from centrifuged washed microsomes was suspended in sucrose-phosphate and was used as microsomal fraction (microsomes). Protein was determined by modified Lowry's method [22] with crystalline bovine serum albumin as standard. The 10,000*g* supernatant, cytosol and microsomes were then adjusted to contain 15 mg protein/ml. The subcellular fractions were at times stored at −20°C, and such samples retained enzymatic activity unchanged for weeks. The homogenates and subcellular preparations from HeLa cells, perfused livers, and livers from pretreated animals were prepared as described above for control animals. For rat liver perfusion, the rats were anesthetized and kept anesthetized for the entire procedure carried out *in situ* according to Miller [23] with sucrose-phosphate as perfusion medium.

Irreversible binding assay

Subcellular fractions were incubated with [^{14}C]-Ledakrin in the presence of NADPH-regenerating system under either air or deoxygenated nitrogen. The complete incubation mixture consisted of a subcellular preparation (0.25–2.5 mg protein/ml), 1 mM NADPH (or 1mM NADP plus 1.0 U/ml of glucose-6-phosphate dehydrogenase), 10 mM glucose-6-phosphate, 12.5 mM nicotinamide, 0.1–0.4 mM [^{14}C]-Ledakrin, 10 mM $MgCl_2$ in 50 mM sodium phosphate buffer, pH 7.4. After 10–15 min of preincubation at 37° without substrate, the enzymatic reaction was initiated by the addition of [^{14}C]-Ledakrin and the mixture was then incubated at 37° for varying times. Control mixtures, used to assay for non-enzymatic irreversible binding, included the appropriate amount of protein from boiled subcellular fraction (100°, 10 min), in place of the intact subcellular fraction. The reaction was terminated by spotting 100 μl aliquots onto Whatman GF/C glass-fibre filter before being dropped into 10 ml/filter of ice-cold 5% trichloroacetic acid (TCA). In this step the enzymatic reaction was stopped, the macromolecules precipitated, and radioactivity reversibly bound to protein roughly extracted. In some experiments, the filters were presoaked with bovine serum albumin (10 mg/ml sucrose-phosphate) prior to the spotting of aliquots of reaction mixture onto filters. Since no significant difference in irreversible binding and/or quantitative recovery of protein had been found, the albumin presoaking was omitted in routine assays. The filters were then extracted in the same beaker twice with 10 ml/filter of ice-cold 5% TCA, and finally once with 10 ml/filter of ethanol. All extraction steps were tested for the radioactivity remaining on the filters in the experiments preceding routine assays, radioactivities were determined before and after every step. After the last ethanol extraction no further radioactivity could be removed from the macromolecules, even if the complete extraction procedure was repeated. The radioactivity bound with the subcellular macromolecules remained constant after these extraction procedures. Therefore, this extraction method removes the original Ledakrin as well as all metabolites reversibly bound with the macromolecules. Protein loss was negligibly low during the procedure. The extraction with ether, performed in test experiments, was omitted in routine assays since this step neither removed any further radioactivity nor improved counting efficiency. After extractions, the filters were placed each in 10 ml of the toluene counting solution (0.4% PPO, 0.01% POPOP) and counted in a Nuclear Chicago Isocap 300 Liquid Scintillation Counter. All samples were counted for a sufficiently long time so that those with the lowest radioactivity were accurate to ± 1%. Radioactivity was corrected for background and quenching by internal or external standarization. From the specific radioactivity and the protein content in the incubation mixture, nmoles of Ledakrin metabolites irreversibly bound per mg protein were calculated. Irreversible binding with subcellular macromolecules was finally expressed as overall irreversible binding (enzymatic plus non-enzymatic reaction) minus non-enzymatic irreversible binding (boiled subcellular fraction-assay), if not otherwise stated.

N-*Arylhydroxylamine determinations*

The standard incubation mixture (1.0 ml) for *N*-arylhydroxylamine determinations assays consisted of 0.2 mM Ledakrin, 1.5 mg of subcellular fraction protein from control rats, 1 mM NADPH, 10 mM glucose-6-phosphate, 12.5 mM nicotinamide, 10 mM $MgCl_2$ in 50 mM sodium phosphate buffer, pH 7.4. The incubation time was 90 min. The reaction was terminated by the addition of 2 vol. of ethanol under nitrogen followed by overlayering with liquid paraffin. After centrifugation at 5000*g* for 15 min, the supernatant was analyzed for *N*-hydroxylated metabolites by the colorimetric method of Boyland and Nery [24]. Pentacyanoamine ferroate was prepared according to Zucker and Nason [25]. The absorbance of complexes was read at 540 nm in 1 cm Silica cells with a Varian Techtron Model 635 D Spectrophotometer (overall reaction values against non-enzymatic reaction blank). However, the quantities of *N*-arylhydroxylamines formed were not determined, because neither hydroxylamine nor amine were available as standards for measurement.

RESULTS AND DISCUSSION

Incubation of [^{14}C]-Ledakrin with subcellular fractions of either rat liver or HeLa cells (Table 1) leads to an irreversible binding of as much as about 30 per cent of Ledakrin radioactivity with subcellular macromolecules after exhaustive extraction with cold trichloroacetic acid, ethanol and ether. The binding seems to be covalent. Only an insignificant portion of the drug is bound with macromolecules if a boiled subcellular fraction was used (0.8 nmolc/mg protein, 3%; Table 2) instead of an intact fraction (25 nmoles/mg protein, 100%; Table 2). The difference between irreversible binding with macromolecules of Ledakrin in the presence of intact and heat-inactivated enzymatic subcellular fractions indicates that the metabolites of Ledakrin rather than Ledakrin itself are responsible for the irre-

Table 1. Irreversible macromolecule binding of [^{14}C]-Ledakrin (0.2 mM) catalyzed by subcellular fractions (1 mg protein/ml) from rat liver or HeLa cells in presence of NADPH-regenerating system after 90 min incubation under air

	Total Ledakrin radioactivity irreversibly bound with macromolecules (nmoles/mg protein)		
Assay	10,000 *g* supernatant	cytosol	microsomes
rat liver homogenate	56.5 ± 0.9 (n = 18)	5.1 ± 0.7 (n = 18)	9.6 ± 0.7 (n = 18)
HeLa cells homogenate	12.9 ± 0.8 (n = 9)	10.7 ± 0.6 (n = 9)	7.2 ± 0.6 (n = 9)

versible binding with macromolecules *in vitro*. Post-mitochondrial supernatant (10,000*g* supernatant), cytosol, and microsomes of either rat liver or HeLa cells are all capable of metabolizing Ledakrin and produce intermediates irreversibly bound with macromolecules (Table 1). The amount of Ledakrin metabolites bound is very high *in vitro* and can be detected either if it is determined directly in the acid-insoluble macromolecules precipitate (see Materials and Methods) or can be calculated from recovery studies. The values for HeLa cells should not be quantitatively compared with rat liver subcellular protein experiments values, because the dependence of the irreversible macromolecule binding of Ledakrin radioactivity on e.g. protein or substrate concentration was not studied in detail for HeLa cells. The metabolism of Ledakrin leading to irreversible binding with macromolecules *in vitro* catalyzed by liver subcellular fractions isolated from perfused livers and from non-perfused livers was compared utilizing the NADPH-generating system. Examination of data (not shown) for conversion of Ledakrin to its reactive metabolites revealed no differences in the metabolism between two groups. The irreversible macromolecule binding of Ledakrin metabolites depends on the protein content of any rat liver fraction in the incubation mixture (Fig. 1). For the incubations under air, saturation is reached with 1.5 mg protein/ml or 2.5 mg protein/ml for 10,000*g* supernatant or microsomes, respectively. These saturation curves are very similar to the curve which demonstrates irreversible binding with microsomal protein of ethynylestradiol [26], estradiol [27], acetaminophen [28], imipramine [29], halothane [30] and chloramphenicol [31]. Therefore, irreversible macromolecule binding of xenobiotics and also of Ledakrin *in vitro* under aerobic conditions is limited by the microsomal or 10,000*g* supernatant protein concentrations, as is well known for microsomal hydroxylation reactions [32]. On the other hand, unlike the irreversible binding under air, the saturation is not observed for the incubations under anaerobic conditions, and the latter binding is almost linear between 0.25 and 2.5 mg protein/ml [Fig. 1].

Among three subcellular fractions studied the highest binding is attributed to experiments with post-mitochondrial supernatant containing both microsomes and cytosol, under both aerobic and anaerobic conditions (Fig. 1). These results infer that microsomes and cytosol can cooperate in the

Table 2. Total (enzymatic plus non-enzymatic) irreversible macromolecule binding of [^{14}C]-Ledakrin (0.2 mM) catalyzed by subcellular fractions (2.5 mg protein/ml) from rat liver in presence of NADPH after 90 min incubation under air

	Total Ledakrin radioactivity irreversibly bound with macromolecules (mmoles/mg protein)		
Assay	10,000 *g* supernatant	cytosol	microsomes
Complete*	24.9 ± 0.2 (n = 12)	15.4 ± 0.2 (n = 12)	24.9 ± 0.3 (n = 12)
With boiled subcellular protein	0.8 ± 0.2 (n = 12)	0.7 ± 0.2 (n = 12)	0.8 ± 0.2 (n = 12)
Without NADPH	0.8 ± 0.2 (n = 12)	0.7 ± 0.2 (n = 12)	0.8 ± 0.2 (n = 12)
With 0.5 mM NADPH	13.2 ± 0.3 (n = 6)	8.0 ± 0.3 (n = 6)	13.0 ± 0.4 (n = 6)
With 1 mM NADPH	24.9 ± 0.2 (n = 12)	15.4 ± 0.2 (n = 12)	24.9 ± 0.3 (n = 12)
With 1.5 mM NADPH	32.3 ± 0.5 (n = 6)	18.6 ± 0.3 (n = 6)	33.6 ± 0.4 (n = 6)

* With NADPH-regenerating system containing 1 mM NADPH and intact subcellular fraction.

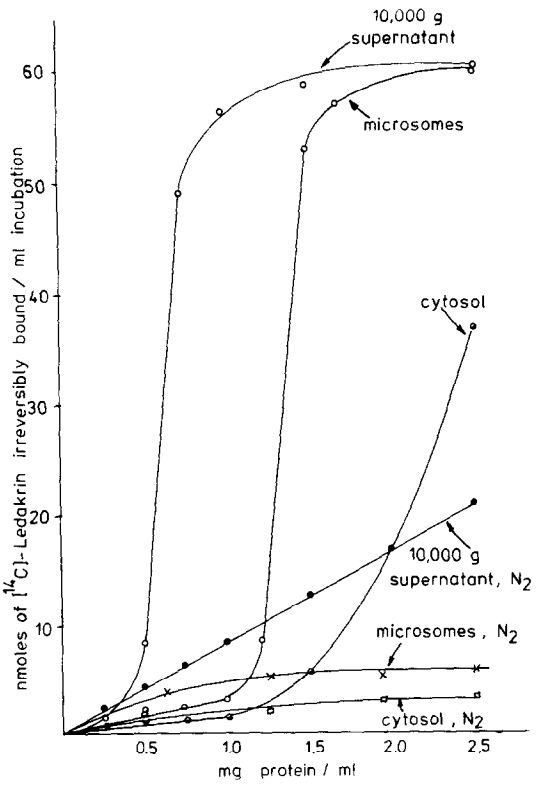

Fig. 1. Irreversible macromolecule binding of [^{14}C]-Ledakrin (0.2 mM) catalyzed by subcellular fractions from control rat liver in presence of NADPH-regenerating system after 90 min incubation under aerobic or anaerobic conditions. Dependence on the subcellular protein concentration.

metabolism of Ledakrin resulting in the formation of irreversibly bound material. The existence of several distinct proteins acting in this metabolism of Ledakrin in the manner suggested for some exogenous or endogenous substrates [33] seems likely. The latter active proteins, although isolated from the cytosol fraction, were reported to be in fact easily dissociable peripheral microsomal proteins directing the transport of intermediates in metabolism of xenobiotics from one specific site or compartment within the membrane system to another [34].

Binding of Ledakrin radioactivity with subcellular macromolecules is also a time dependent process (Fig. 2) which is linearly increased between 5 and 60 min, complete after 90 min and then remaining at a plateau. This might be regarded as a further indication for an enzyme reaction being involved. The incubation time of 90 min was used in routine assays. The irreversible macromolecule binding of Ledakrin metabolites is dependent on the substrate concentration (Fig. 3). The Ledakrin concentration of 0.2 mM was chosen for standard experiments as close to saturation conditions. The binding is directly proportional to the concentration of NADPH which was added to the incubation system instead of the NADPH-regenerating system (Table 2). Concordantly, only an insignificant portion of Ledakrin is bound with macromolecules if NADPH in our complete system was omitted (Table 2), and the drug radioactivity bound amounted only to 3 per cent (0.8 nmole/mg protein) of the total metabolites of Ledakrin bound under optimal conditions (25 nmoles/mg protein). NADPH-dependency is further evidence for an enzyme reaction being involved in the irreversible binding of Ledakrin with subcellular macromolecules. The dependence of the binding of the drug radioactivity under nitrogen atmosphere on substrate concentration, NADPH, as well as on time of incubation, is very similar to that hitherto shown for incubations under air (data not shown).

To obtain information concerning the enzymes mediating the macromolecule binding of Ledakrin *in vitro*, microsomes and cytosol from not-pretreated rats and from rats which had been pretreated with 3-methylcholanthrene or phenobarbital were compared for their ability to catalyze irreversible binding of the drug radioactivity. The macromolecule binding of Ledakrin is significantly increased in microsomes from phenobarbital treated rats while that catalyzed by cytosol obtained from the same livers is unchanged (Table 3). Treatment of animals with phenobarbital increases cytochrome P-450 content without changing the spectral characteristics of microsomes, whereas treatment with polycyclic hydrocarbons such as 3-methylcholanthrene gives rise to a new predominant form with unique spectral characteristics, known as cytochrome P_1-450 [35] or P-448 [36]. Therefore, in general, higher binding

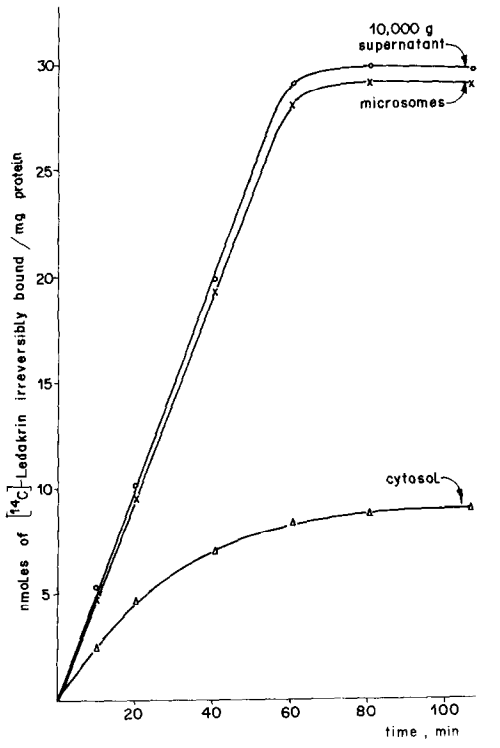

Fig. 2. Irreversible macromolecule binding of [^{14}C]-Ledakrin (0.2 mM) catalyzed by subcellular fractions from control rat liver (2 mg protein/ml) in presence of NADPH-regenerating system under aerobic conditions. Dependence on the time of incubation.

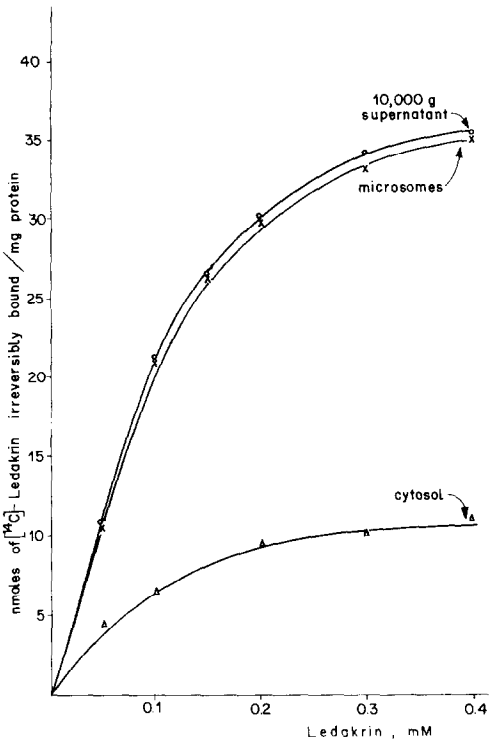

Fig. 3. Irreversible macromolecule binding of [^{14}C]-Ledakrin catalyzed by subcellular fractions from control rat liver (2 mg protein/ml) in presence of NADPH-regenerating systems after 90 min incubation under aerobic conditions. Dependence on the Ledakrin concentration.

observed with pretreated rats can be attributed to enhanced drug-metabolizing activity in the liver induced by phenobarbital or 3-methylcholanthrene.

Table 3. Irreversible macromolecule binding of [^{14}C]-Ledakrin (0.2 mM) catalyzed by rat liver subcellular fractions (2.5 mg protein/ml) in presence of NADPH-regenerating system after 90 min incubation under air*

Enzymatic fraction	Ledakrin metabolites irreversibly bound (per cent control)‡	
	Microsomes (%)	Cytosol (%)
Without pretreatment (control†)	100 ± 7	100 ± 11
With phenobarbital pretreatment	192 ± 17	94 ± 10
With 3-methylcholanthrene pretreatment	186 ± 18	106 ± 9

* Liver subcellular fractions from not-pretreated (control) or pretreated rats were prepared as described in Materials and Methods. Values are the mean of at least three rats in any experiment.

† Not-pretreated rats experiments were assumed as control experiments.

‡ Values are for $n = 6$.

The dependence of the binding of Ledakrin radioactivity with subcellular macromolecules of post-mitochondrial supernatant or microsomes on microsomal enzyme, on substrate concentration, oxygen and NADPH, as well as the induction of this reaction with phenobarbital or 3-methylcholanthrene pretreatment, indicate that the oxidative binding of metabolites of Ledakrin is catalyzed by a mixed-function oxidases, probably by the unspecific drug metabolizing system involving cytochrome P-450 of liver microsomes.

Based on these results, to account for irreversible binding of Ledakrin through a metabolic activation

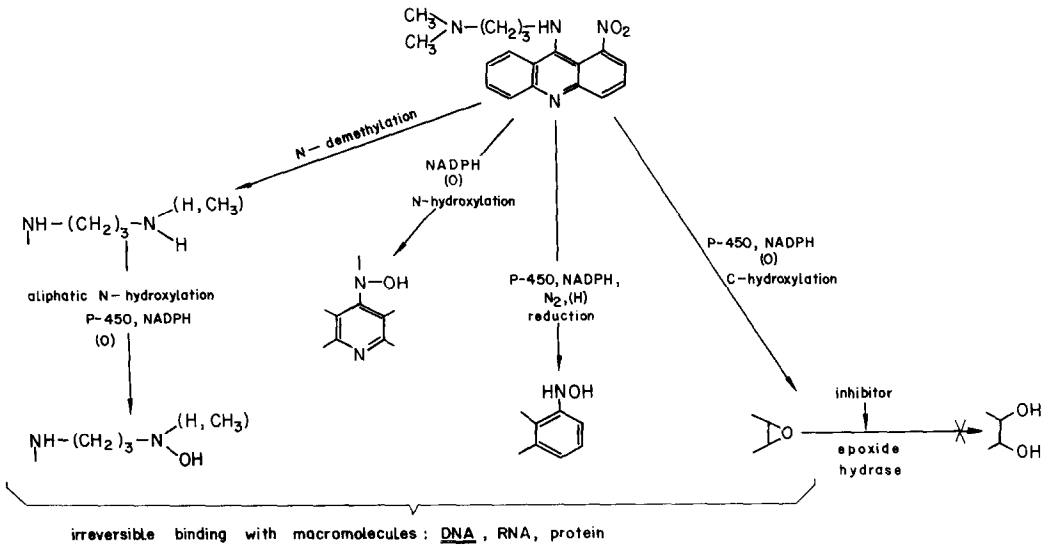

Fig. 4. Possible metabolic activation of Ledakrin catalyzed by subcellular fractions from either rat liver or HeLa cells leading to irreversible macromolecule binding of the drug.

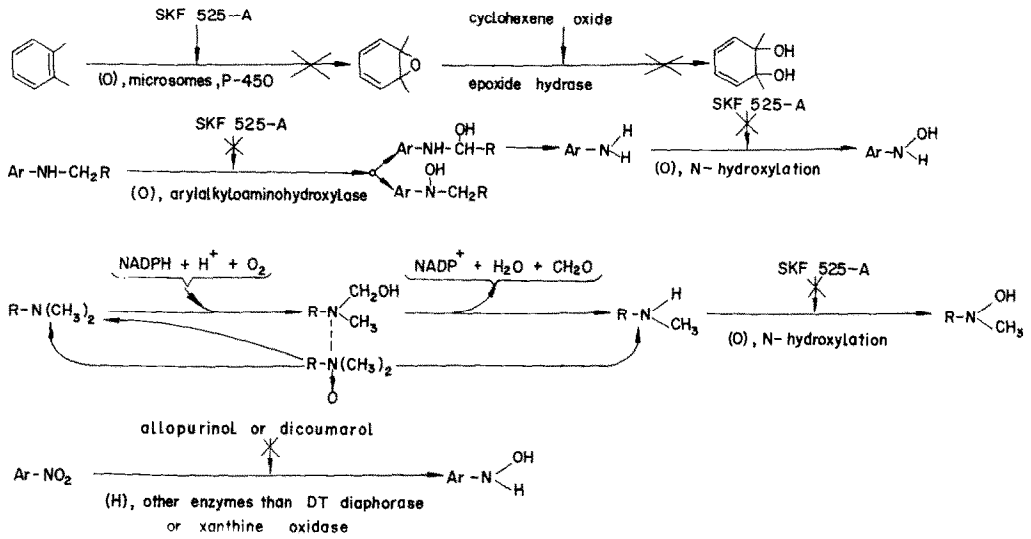

Fig. 5. Inhibitors of xenobiotic metabolic pathways.

under aerobic conditions *in vitro*, three potential pathways were considered most likely (Fig. 4): (1) C-hydroxylation [37–40], (2) *N*-alkylhydroxylamine formation [41] and (3). aromatic *N*-hydroxylation [17–19]. All pathways are oxygen- and NADPH-requiring processes, can be mediated by microsomal cytochrome P-450 system or other complex enzymes present in subcellular fractions studied, and each fulfills the requirement of retaining the entire molecule radioactivity-labeled acridine ring upon irreversible binding. To account for irreversible macromolecule binding of Ledakrin under highly anaerobic conditions, an intermediate arising from the enzymatic reduction was considered. A pathway leading through *N*-arylhydroxylamine, derived from the nitro group reduction, appeared the most likely possibility (reviewed e.g. in Refs. 17–19) (Fig. 4). To verify that all four postulated pathways are involved in irreversible macromolecule binding of Ledakrin *in vitro*, we examined the binding of the activated drug in the presence of specific inhibitors of different subcellular enzymes (Fig. 5).

To confirm that an arene oxide of the acridine ring is the reactive intermediate in irreversible binding, the effect of cyclohexene oxide, a potent inhibitor of epoxide hydrase [42], on the macromolecule binding was studied. The postulated epoxide, if formed, could be converted according to its stability to the corresponding phenol or further metabolized to a chemically inert dihydrodiol compound [43] by epoxide hydrase [42–44]. A concentration of 2 mM cyclohexene oxide causes a nearly 2-fold increase in irreversible binding (Table 4). The thermal inactivation of epoxide hydrase [45] also produces an augmentation of the binding of 30 per cent (Table 4). Hence, as has been shown for the covalent binding of polycyclic aromatic hydrocarbons with cellular macromolecules [46], we evidenced that by preventing the enzymatic hydration of an acridine epoxide the steady-state level of this reactive intermediate

Table 4. Effect of inhibitors of epoxide hydrase on irreversible macromolecule binding of [^{14}C]-Ledakrin (0.2 mM) catalyzed by rat liver subcellular fractions (2.5 mg protein/ml) in presence of NADPH-regenerating system after 90 min incubation under air

	Ledakrin metabolites irreversibly bound (per cent control)*	
Assay	10,000 *g* supernatant (%)	Microsomes (%)
Without inhibitor (control)†	100 ± 5	100 ± 9
With 2 mM cyclohexene oxide	131 ± 7	189 ± 11
With preincubation at 55° for 5 min	110 ± 2	130 ± 5

* Values are for $n = 12$.

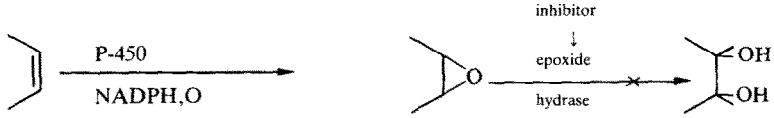

Table 5. Irreversible macromolecule binding of [^{14}C]-Ledakrin (0.2 mM) catalyzed by subcellular fractions (2 mg protein/ml) from rat liver in presence of NADPH-regenerating system, after 90 min incubation under air. Effect of SKF 525-A, reduced glutathione and amino acid derivatives

	Ledakrin metabolites irreversibly bound (per cent control†)		
Assay	10,000 *g* supernatant (%)	Cytosol (%)	Microsomes (%)
Without inhibitor (control†)	100 ± 3 (n = 12)	100 ± 4 (n = 12)	100 ± 6 (n = 12)
With SKF 525-A (1 mM)	95 ± 2 (n = 9)	—*	94 ± 3 (n = 9)
With reduced glutathione (1 mM)	56 ± 3 (n = 9)	45 ± 7 (n = 9)	51 ± 8 (n = 9)
With N-acetyl-L-alanine (1 mM)	101 ± 5 (n = 6)	97 ± 7 (n = 6)	105 ± 11 (n = 6)
With glycylglycine ethyl ester · HCL (1mM)	98 ± 3 (n = 6)	106 ± 5 (n = 6)	108 ± 4 (n = 6)
With *N*-acetyl-L-tyrosine ethyl ester (1 mM)	99 ± 5 (n = 6)	63 ± 3 (n = 6)	121 ± 4 (n = 6)

* Not assayed.

formed during the binding reaction had been increased and resulted in a greater degree of oxidative irreversible binding of activated Ledakrin. To evaluate the importance of postulated *N*-hydroxylation reactions (Fig. 4) in the process of oxidative drug activation, we studied the effect of SKF 525-A on the Ledakrin radioactivity binding with macromolecules *in vitro*. The specific inhibitor of microsomal C-oxidation wherein the epoxidation step is involved, SKF 525-A [45], does not significantly diminish the binding of Ledakrin metabolites with macromolecules of either post-mitochondrial supernatant or microsomal fraction of rat liver (Table 5). The ineffectiveness of SKF 525-A to decrease the irreversible binding is indirect evidence that besides microsomal C-oxidation other oxidative activations are involved. The increase of the formation of other than arene oxide hydroxylation product might counteract the decrease of C-hydroxylation reaction caused by SKF 525-A. The pathways leading to *N*-hydroxylamines (Fig. 4) seems likely since *N*-oxidations are not inhibited by SKF 525-A [42, 45, 47], as is the case for other reactions mediated by microsomal cytochrome P-450 system (Fig. 5). An aliphatic *N*-hydroxylation of the amino group of Ledakrin side chain can be the prerequisite for that undefined binding, especially since the *N*-alkylhydroxylamine formations are not always cytochrome P-450 dependent [41]. However, the direct evidence for the formation of *N*-alkylhydroxylamine intermediate of Ledakrin deserves further investigation. The formation of *N*-alkylhydroxylamine should require prior demethylathion(s) at the amine ω-nitrogen of Ledakrin side chain, followed by aliphatic *N*-hydroxylation. The demethylation of one methyl group of the drug will lead to desmethyl-Ledakrin, and further demethylation to the amine, desdimethyl-Ledakrin. Neither compound should react with nucleophilic subcellular receptors in its own right. Among the possible metabolic activations of Ledakrin under aerobic conditions (Fig. 4), the transformation of Ar-NH-CH_2— into Ar-NOH-CH_2— by *N*-oxidation, leading to an arylhydroksylamine is considered since arylhydroxylamines form under air with all subcellular fractions studied, as determined colorimetrically (Table 7). The aromatic *N*-hydroxylation discussed may be preceded by a dealkylation of the aliphatic side chain linked to the nitrogen atom at the position 9 of acridine ring. The latter possibility can not, however, be either supported or ruled out in our experiments with the drug molecule labeled with carbon-14 in the acridine ring.

To account for irreversible binding of Ledakrin under highly anaerobic conditions, a *N*-arylhydroxylamine arising from the nitro group reduction seems the most likely to be an intermediate determined colorimetrically [Table 7]. The metabolism of the nitro group of Ledakrin to its parent *N*-hydroxylamine is directly related to the irreversible binding of the drug metabolites with subcellular macromolecules *in vitro* under nitrogen (Table 7). Thus, the increase in irreversible binding appears to be dependent on the enhanced formation of *N*-arylhydroxylamine under anaerobic conditions. The reduction activity does not seem to be due to either DT diaphorase [48] or xanthine oxidase activity [49–51]. Neither dicoumarol, a potent inhibitor of DT diaphorase [52], nor allopurinol, an inhibitor of xanthine oxidase [49], inhibited irreversible binding of Ledakrin radioactivity with macromolecules of any subcellular fraction in incubations under nitrogen (Table 6). The participation of the cytochrome P-450 is also not an absolute requirement for the reductive binding of metabolites of Ledakrin with subcellular macromolecules, since the activated drug binds with cytosol macromolecules too (Fig. 1). Therefore, the involvement of other enzymes such as NADPH-cytochrome P-450 reductase [53], NADPH-cytochrome *c* reductase [49–51, 54, 55], aldehyde oxidase [48], or other unknown liver enzymes is to be considered as mediating the reductive activation of Ledakrin *in vitro*. To obtain infor-

Table 6. Irreversible macromolecule binding of [^{14}C]-Ledakrin (0.2 mM) catalyzed by post-mitochondrial supernatant (2 mg protein/ml) from rat liver in presence of NADPH-regenerating system after 90 min incubation under nitrogen. Effect of inhibitors of oxidoreductases and reduced glutathione

Assay	Ledakrin metabolites irreversibly bound (per cent control*)
Without inhibitor (control†)	100 ± 6
With 0.05 mM allopurinol	88 ± 13
With 0.05 mM dicoumarol	108 ± 3
With 1 mM reduced glutathione	66 ± 11

* Values for $n = 6$.

mation concerning the nature of the target in macromolecules, the effect of several compounds known to trap reactive metabolites were studied. Reduced glutathione decreases significantly the irreversible macromolecule binding of metabolites of Ledakrin (Tables 5 and 6). These results infer that the active metabolites irreversibly bound with subcellular macromolecules are electrophilic agents since reduced glutathione is a nucleophilic compound capable of reacting with electrophilic alkylating agents. The dipeptides with other than −SH free potential trapping groups almost negligibly block the oxidative binding of the activated drug (Table 5). Therefore, reduced glutathione might remove the reactive Ledakrin metabolite(s) formed most probably by establishing a chemically stable thioether bond. Thioethers arise from the reaction of metabolites of xenobiotics with SH-compounds, especially reduced glutathione [42, 56]. Thioethers are usually formed enzymatically by glutathione transferases [56, 57], but non-enzymatic conjugations of electrophilic intermediates with glutathione have also been observed [43]. The latter seems to be the case with Ledakrin; liver cytosol glutathione transferase, which catalyzes direct conjugation of glutathione and xenobiotics [42, 56, 57], does not appear essential for the irreversible binding of the activated drug with glutathione since the binding with cytosol macromolecules was pronounced to the same extent as compared to other subcellular fractions in the presence of reduced glutathione (Table 5).

If bovine serum albumin was added to microsomal protein incubation system, Ledakrin radioactivity was irreversibly bound with this protein too (data not shown). This binding could also be inhibited by reduced glutathione in accordance with the experiments measuring only microsomal protein binding. Thus, the binding of the drug metabolites with albumin itself may be the reason that albumin decreases their binding with microsomal macromolecules.

The present study evidences on the metabolic activation of Ledakrin *in vitro* as being an essential prerequisite for irreversible binding of the drug with subcellular macromolecules of either rat liver or HeLa cells *in vitro*, and thereby supports our previous findings based on the *in vivo* experiments with 1-nitroacridines [10, 12–16]. The intermediates from such indirect-acting compounds may interact covalently at nucleophilic sites in cellular nucleic acids

Table 7. The relationship between the formation of *N*-arylhydroxylamines and the irreversible macromolecule binding of [^{14}C]-Ledakrin (0.2 mM) by the action of subcellular fractions (1.5 mg protein/ml) from rat liver homogenates in the presence of NADPH-regenerating system after 90 min incubation either under air or nitrogen

	Assay	Subcellular fraction		
		10,000 *g* supernatant	Microsomes	Cytosol
Under nitrogen	*N*-arylhydroxylamine formation (E_{540})	0.583 ± 0.039 ($n = 4$)	0.400 ± 0.025 ($n = 4$)	0.375 ± 0.013 ($n = 4$)
	Ledakrin metabolites irreversibly bound (nmoles/ml)	12.8 ± 0.6 ($n = 12$)	6.0 ± 0.5 ($n = 12$)	3.3 ± 0.3 ($n = 12$)
Under air	*N*-arylhydroxylamine formation (E_{540})	0.508 ± 0.25 ($n = 4$)	0.256 ± 0.018 ($n = 4$)	0.130 ± 0.036 ($n = 4$)
	Ledakrin metabolites irreversibly bound (nmoles/ml)	59.5 ± 0.8 ($n = 18$)	53.0 ± 0.9 ($n = 18$)	6.1 ± 0.5 ($n = 18$)

and proteins, and one or more of these processes are generally assumed to be responsible for their antitumor, carcinogenic or mutagenic activity, e.g. Refs. 17–19. The results presented herein indicate the possibility of formation of four potential binding sites in the Ledakrin molecule *in vivo*. The postulated reactive electrophilic metabolites of Ledakrin, if in fact formed in the cell, seem capable of reacting irreversibly with subcellular macromolecules in their own right. However, it appears likely that these intermediates might be in fact 'proximate metabolites' of the drug, being themselves the species converted in the cell to 'ultimate metabolites' of Ledakrin to react irreversibly with DNA,RNA and/or protein. The oxidation and reduction products, the *N*-hydroxylamines, might be further activated *in vivo* by esterification, as has been proposed for *N*-hydroxyl-2-acetaminofluorene [17] or 4-*N*-hydroxylaminoquinoline [58]. Besides the detected *N*-arylohydroxylamine formed by a reduction of the nitro group, there may also exist such reduced intermediates as nitroso compound or amine [18, 54, 55]. The reductive binding of Ledakrin may be thus alternatively mediated by the mechanism involving formation of a free radical intermediate or nitroso derivative, as has been postulated for some carcinogenic nitro compounds [59]. The formation of an arene oxide in acridine nucleus may be the initial step in activating Ledakrin *in vivo* via C-hydroxylation pathway. It is likely that the postulated acridine epoxide, besides reacting with cellular macromolecules directly, might itself be an intermediate converted in the cell into other electrophiles [60], as has been shown for some carcinogens [61] or potential antileukemic agents [62]. Therefore, it appears finally that each of the Ledakrin metabolites postulated herein can undergo in the cell further activations to 'ultimate metabolites' capable of binding with cellular macromolecules or of interacting irreversibly with DNA, RNA and/or protein in its own right.

The postulated glutathione conjugate of Ledakrin metabolite(s) may be quantitatively and/or qualitatively of significant importance in metabolism of the drug undergone in cells with high glutathione levels. The conjugation of glutathione with proximate and/or ultimate metabolite(s) of Ledakrin may therein represent a detoxification mechanism for the drug, as it has been found for e.g. bromobenzene when reduced glutathione protected the liver by reacting with a toxic active metabolite [63]. The finding that Ledakrin metabolites were irreversibly bound with a soluble protein like cytosol or albumin added to the microsomal incubation mixture, led us to assume that the reactive metabolite(s), if formed, may be stable enough in the cell to leave the microsomal membrane to react with cellular macromolecules outside the endoplasmic reticulum since binding of drug metabolite(s) with a soluble protein catalyzed by microsomal enzyme can be viewed as an *in vivo* model.

The results presented in this report indicate the possibility of formation of reactive Ledakrin metabolites *in vivo* via both oxidative and reductive pathways. If all activations of the drug herein postulated do occur in a Ledakrin-sensitive cell, it can be presumed that six metabolites of Ledakrin bearing each two reactive binding sites may appear in the cell. Our results of the *in vitro* experiments can not however infer that reactive metabolite of the drug which binds bifunctionally with cellular DNA thereby preventing strand separation. Hence, the mechanism of cross-linking of cellular DNA by metabolically activated Ledakrin or other 1-nitroacridines still deserves further studies. Our previous *in vivo* studies suggested a location of the nitro group in the position 1 of acridine nucleus as being an essential prerequisite for the biological activity of nitroacridines [4–6]. The unique ability of 1-nitroacridines to cross-link DNA of both mammalian and bacterial cells [12, 14, 16] appears to support the latter finding. Crystal and molecular structure of Ledakrin, as solved by X-ray analysis, is also atypical [64] as compared with acridine derivative structures [65–71] which recently include the structure of the 2-nitro analogue of the drug [72]. Therefore, one could expect that detailed studies on the metabolism of Ledakrin or other 1-nitroacridines resulting in an irreversibly bound macromolecular material should help to understand the unique role of a location of the nitro group in the position 1 for the biological action of nitroacridines. The results of *in vitro* experiments with Ledakrin presented in this report infer that the oxidations appear to be responsible, rather than reductive pathway, for the total (mono-plus bi- functional) irreversible macromolecule binding of Ledakrin metabolites *in vivo*, because the apparently higher binding of Ledakrin radioactivity is regarded *in vitro* to aerobic conditions (see e.g. Fig. 1) and on the other the oxygen tension in liver and other organs is at a significant level [73]. Thus, all the hitherto presented *in vivo* and *in vitro* findings imply that to account for the unique role of a location of the nitro group in an acridine nucleus in the position 1, two explanations seem likely: (1) a reductive metabolism of the nitro group in the position 1, although presumably lower *in vivo* than oxidative pathways, may lead to formation of drug–macromolecule covalent bonds of particularly great importance for a biological effect of Ledakrin or other 1-nitroacridines, and/or (2) the nitro group in the position 1 may activate acridine molecules for either the overall or particular metabolic pathway(s) leading to the formation of other than 1-N-hydroxylamine reactive binding site(s), e.g. epoxide or *N*-hydroxylamine(s). If the activity of cytochrome P-450 system-linked enzymes is in fact responsible for the total (mono-plus bifunctional) Ledrakrin irreversible binding *in vivo*, it can be presumed that Ledrakrin may be selectively cytotoxic against tissues or organs bearing high cytochrone P-450 content such as liver, lung, spleen and kidney. Nevertheless, some processes, like e.g. epoxide hydrase activity in the endoplasmic reticulum and/or glutathione level in the cell, could diminish the extent of the macromolecule binding of Ledakrin by detoxification of an active drug metabolite formed before it can react with a target macromolecule. It seems also interesting to prove Ledakrin against tumors originating from the organs with high cytochrome P-450 content and/or as an inhibitor of metastasis to these organs. The irreversible macromolecule binding of Ledakrin

metabolites deserves further investigation to find out the true mechanism of action of the drug.

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