REACTIONS OF 2-FURYLETHYLENES WITH THIOLS *in vivo**

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The reaction of furylethylenes with thiols proceeds also *in vivo*. This has been proved with Ehrlich ascitic carcinoma cells and yeast under steady-state conditions by determination of SH groups employing the Ellman reagent. 1-(5-Nitro-2-furyl)-2-nitroethylene caused a considerable decrease of intracellular thiols content proportional to the amount used. A correlation between the degree of modification of SH groups *in vivo* and the reactivity towards thiols estimated under the *in vivo* conditions was found. The ability of furylethylenes to react with SH groups is decisive for their biologic activity.

Nucleophilic functional groups play a significant role in the chemism of biological systems. Especially the SH groups[1-3] are of a dominant position in chemical transformations proceeding in the living nature. A great number of enzymes is known, the catalytic activity of which is subject to the presence of SH groups belonging to the embodied cysteine. These enzymes take part in such important processes as glycolysis, oxidative and photosynthetic phosphorylation, transport of nutrients, microsomal hydroxylations, proteosynthesis, synthesis of nucleic acids *etc.*[1]. Nonetheless, the non-protein thiols, as *e.g.* cysteine, glutathione, dihydrolipoic acid, coenzyme A and further low-molecular SH-containing compounds are of great importance in the metabolism[1]. It is, therefore, understandable that the effect of many biologically active substances is just due to the ability to modify chemically the thiol groups of the cell[4]. This paper provides evidence that derivatives of furylethylene belong to these substances and can be regarded as a continuation of our programme on reactions furylethylenes with low-molecular model thiols and thiolproteins *in vitro*[5-7]. The goal of it is to prove that these reactions are of use also *in vivo*, and are decisive from the standpoint of the nature of cytotoxic activity of furylethylenes.

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Abbreviations: DTNB Ellman reagent, EAC Ehrlich ascitic carcinoma, GSH reduced glutathione, TRIS tris(hydroxymethyl)aminomethane.

EXPERIMENTAL

Chemicals. Furylethylenes were synthesized in the Department of Organic Chemistry, Slovak Institute of Technology, Bratislava. The stock solutions in dimethyl sulfoxide are considered to be the most favourable for the study of interactions of biologically active substances with various types of prokaryotic and eukaryotic cells[8]. The finale concentration of dimethyl sulfoxide in incubation mixtures never exceeded 1%. The reduced glutathione (GSH, γ-glutamylcysteinylglycine) was the commercial product of Sigma (USA).

Yeasts, EAC cells and mitochondria. Saccharomyces cerevisiae XII (Czechoslovak Collection of Yeasts, CCY 21-4-13), *S. cerevisiae* XIIa (CCY 21-4-19) and *Candida albicans* Pn 10 (CCY 29-3-91) were those strains used as yeast models. The strains were cultivated on a sweet wort agar and passaged every 14 days. A synthetic medium[9] containing glucose as the carbon source and ammonium sulfate as a nitrogen source, inorganic salts, trace elements and vitamines was employed for cultivation at 28°C on reciprocal shaking machine in 0·5 l flask provided with a side neck; the culture medium formed 1/5 of this volume. The cultivation was monitored densitometrically. Cultures from the exponential phase of growth were used for investigating the respective compound effects. The EAC cells were obtained from the peritoneal mice cavity on the eighth day after transplantation. They were suspended in a Krebs–Ringer pH 7·4 phosphate medium without Ca^{2+}, but with ascitic serum (2·5%) and glucose (3 mmol/l). Concentration of cells was densitometrically adjusted to $1{\cdot}6 \cdot 10^6$/ml of the medium. Other data were described in the preceding paper[10]. Mitochondria were isolated from the rat liver according to[11], the content of proteins was characterized by the biuret method[12]. Mitochondria were kept in an ice medium containing saccharose (0·25 mol/l), morpholinopropane sulfonate (2 mmol/l), and EDTA (2 mmol/l), pH 7·2.

Manometric measurements were effected with a Warburg apparatus (Medizintechnik, GDR), with 12 vessels under aerobic conditions (100 swings/min) at 25°C. The oxygen consumption or the carbon dioxide production were monitored in a medium containing 0·1 mol/l KH_2PO_4 and 2% of glucose. The final concentration of yeasts was $5 \cdot 10^7$/ml. The measurement was started after temperating the vessels. The measuring process and calculation of the consumed oxygen and carbon dioxide produced was already described in detail[13].

Investigation of respiration of mitochondria. The oxygen consumption expressed in nmol O_2/min per 1 mg of proteins was measured by means of a Clark oxygen electrode[14] in a temperated cell provided with a stirring bar. The rate of respiration, ADP: O quotient and the respiration control ratio as criteria for structural integrity of mitochondria were calculated according to[15]. Employed were mitochondria with an ADP : O quotient greater than 1·3 only and a respiration control ratio (RCR) greater than 3·0. The respiration medium (2 ml) contained saccharose (0·25 mol/l), morpholinopropane sulfonate (12 mmol/l), KH_2PO_4 (10 mmol/l), EDTA (0·2 mmol/l), rotenone (2·5 μmol/l), and succinate (35 μmol/l). The consumption rate of oxygen in the absence and in the presence of ADP (final concentration 160 μmol/l) and 1-(5--nitro-2-furyl)-2-nitroethylene, or its reaction product with reduced glutathione was recorded after addition of mitochondria (final concentration of proteins 1·0 mg/ml).

Determination of the SH *groups content.* The respective furylethylenes (dissolved in dimethyl sulfoxide) were added in the appropriate concentrations to the suspension of yeasts (5 ml, $5 \cdot 10^7$ cells/ml of 0·1 mol/l KH_2PO_4), or EAC cells ($1{\cdot}5 \cdot 10^6$/ml of the Krebs–Ringer phosphate buffer); a pure solvent was used for control (final concentration 1%). After 30 (EAC cells), 60 (yeast), or 120 min (EAC cells) of incubation at 25°C (yeast), or 37°C (EAC cells), the suspension was centrifuged (1 000g, 10 min, 0°C) and the sediments suspended in TRIS (1 ml, 0·2 mol/l, pH 7·2) containing the Ellman reagent (DTNB, 0·4 mg). An ice-cold methanol (4 ml)

was added to the suspension after 10 min, and absorbance of the supernatant was measured at 412 nm after centrifugation. The concentration of SH groups was calculated provided the molar absorption coefficient of the reaction product from Ellman reagent and thiols[16] is 13 600 l. . mol^{-1} cm^{-1} at 412 nm. This procedure serves for determination of total, *i.e.* protein and non-protein SH groups, the estimation error being less than 5%. The non-protein SH groups were determined by addition of 5-sulfosalicylic acid (5%, 15 ml) to the sediment after centrifugation. of the incubation mixture of cells with furylethylenes (40 ml) and standing at 0°C for 90 mind The mixture was centrifuged at 1 000g (0°C, 2 min); TRIS pH 8·9 buffer (0·4 mol/l, 3 ml) and methanolic DTNB (4 mg/ml, 100 μl) were added to the supernatant and the absorbance measures at 412 nm. This method for determination of the total, non-protein and protein sulfhydryl group in yeast and EAC cells has already been described in more detail[17].

RESULTS AND DISCUSSION

As we have already reported[18], furylethylenes reveal a significant biological activity in yeast and animal cells; their action is focused to inhibition of bioenergetic processes, especially of glycolysis and oxidative phosphorylation. Were the ability of furyl-ethylenes to modify the functional sulfhydryl groups of the catalytically active proteins of the appropriate pathway decisive, then the preincubation of compounds with the thiol should result in a decrease, or even in an elimination of their negative impact on biosystems. To verify this assumption, we proposed a series of experiments in which the biological effect of the compound itself and its reaction product with glutathione, as a model thiol substance on the yeast *S. cerevisiae* XIIa, mitochondria isolated from the rat liver, and on the yeast *S. cerevisiae* XII, was characterized. The *S. cevevisiae* XIIa is a respiratory deficient acriflavine mutant of the yeast strain *S. cerevisiae* XII, which gains energy for biologic purposes exclusively by anaerobic degradation of glucose[19] even under aerobic conditions, and therefore, it is suitable for indication of the intervention of bioactive substances in glycolysis. Mitochondria have been employed to demonstrate how the oxidative phosphorylation is influenced, and *S. cerevisiae* XII were such a cell model substance having both fundamental energy generating processes considerably pronounced.

1-(5-Nitro-2-furyl)-2-nitroethylene (*I*) was chosen as a representative of this series. The effects of *I* and its reaction product with glutathione (GSH) on aerobic fermentation of *S. cerevisiae* XIIa were estimated under the steady-state conditions by a manometric monitoring the CO_2 production. No consumption of O_2 has taken place during the measurement of the model reaction. The final concentration of *I* in the incubation medium was $1 . 10^{-4}$ mol/l. To evaluate the effect of reaction product of compound *I* with GSH, the former was at first pre-incubated with GSH ($1 . 10^{-3}$ mol/l) directly in the 0·1M-KH_2PO_4, then glucose and suspension of yeast were added. The 30 min-temperation was followed by the CO_2 formation measument. Results of this experiment are plotted in Fig. 1*a*. As seen, compound *I* is a significant inhibitor of aerobic fermentation of *S. cerevisiae* XII. Preincubation with GSH virtually suspended its effect on the afore-mentioned process.

An analogous experiment was done with mitochondria isolated from rat liver. The effect on O_2-consumption was checked with *I* (final concentration in the medium $5 . 10^{-4}$ mol/l) and its product with GSH. The former was obtained by a 30 min-preincubation of *I* with a 10-fold excess of GSH in the respiration medium at 25°C. Fig. 1*b* shows a remarkable inhibition effect of *I* on the respiration of mitochondria in stage 4 (ref.[15]) on succinate. On the other hand, the reaction mixture of *I* with GSH has the same consumption of O_2 by mitochondria as the control. Also this experiment resulted in a loss of biological activity after reaction with glutathione.

The inhibition of both O_2 consumption and CO_2 production in *S. cerevisiae* XII was practically stopped by the action of *I*, when this was first reacted with GSH

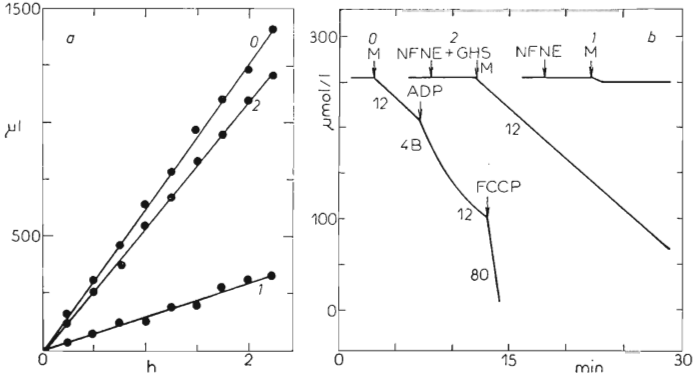

FIG. 1

Aerobic fermentation of *S. cerevisiae* XIIa (*a*) and respiration of the rat liver mitochondria (*b*) in the absence *0* and in the presence of 1-(5-nitro-2-furyl)-2-nitroethylene (NFNE, *1*), or its reaction product with glutathione (NFNE + GSH, 2). Formation of CO_2 by yeast monitored manometrically in a phosphate solution (0·1 mol/l) with 2% of glucose at 25°C. The consumption of O_2 by mitochondria was measured by the Clarke electrode in a succinate (0·035 mmol/l) containing medium at 25°C. Numerals beneath the curves relate to the O_2 consumption in nmol/mg of protein per 1 min. Concentration of the yeast $5 . 10^7$/ml, the amount of mitochondria corresponds to the 1 mg/ml concentration of proteins. The final concentration of furylethylene was $1 . 10^{-4}$ and $5 . 10^{-4}$ mol/l for *a* and *b*, respectively. Glutathione was in both experiments applied in a 10-fold molar excess. The incubation time of reactants was 30 min. Criterion for the functionality of mitochondria was the response on ADP ($1·6 . 10^{-4}$ mol/l) and 4-trifluoromethoxyphenylhydrazonopropanedinitrile (FCCP, $1 . 10^{-7}$ mol/l)

(Fig. 2). The reaction conditions were identical with those employed in experiments with the respiratory deficient mutant *S. cereviaise* XIIa. The incubation time and excess of GSH were chosen in all experiments to meet requirements for an almost quantitative reaction of furylethylene. Glutathione itself does not display any observable effect on any of the tested biological models in the given concentration range.

The results indicate that the biological activity of 1-(5-nitro-2-furyl)-2-nitroethylene and probably also of further furylethylenes is associated with their ability to react with the SH groups of the cell. These reactions were directly indicated by a method enabling to determine the content of SH groups in the cell before and after the action of furylethylenes at concentrations considerably influencing the already mentioned metabolic process of the yeast investigated. The effect of compound *1* on the level of total, *i.e.* of the protein and non-protein groups in *S. cerevisiae* XII and *S. cerevisiae* XIIa shows Fig. 3. The decrease of SH groups with both is proportional to the

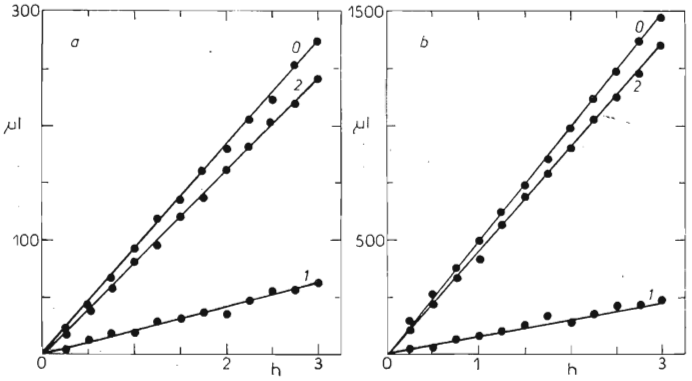

FIG. 2

Consumption of O_2 (*a*) and the formation of CO_2 (*b*) by *S. cerevisiae* XII in the absence *0* and in the presence of 1-(5-nitro-2-furyl)-2-nitroethylene *1*, or its reaction product with glutathione *2*. The exchange of gases was monitored manometrically in a phosphate solution (0·1 mol/l) with 2% of glucose at 25°C. The yeast concentration was $5 \cdot 10^7$ cells/ml, the final concentration of furylethylene $1 \cdot 10^{-4}$ mol/l, of glutathione $1 \cdot 10^{-3}$ mol/l. The incubation time of reactants 30 min

concentration of *I*. The SH groups content was estimated with the Ellman reagent after a 1 h-incubation of the yeast with *I* under steady-state conditions. The calculated content of SH groups in *S. cerevisiae* XII is $4{\cdot}5 \cdot 10^{-8}$ mol per $5 \cdot 10^{7}$ cells and that in *S. cerevisiae* XIIa $3{\cdot}1 \cdot 10^{-8}$ mol per $5 \cdot 10^{7}$ cells. Compound *I* was applied in the highest concentration ($5 \cdot 10^{-4}$ mol/l) in a more than a 10-fold molar excess with respect to SH groups accessible to Ellman reagent. Comparison of these data with the degree of the content of intramolecular thiols decrease could serve, at a statistically greater series of measurements, for a quantitative concept on modification of SH groups of the cell. The protein SH groups contitute the principal portion of the total SH groups content of yeast. This was found when determining the content of low-molecular SH groups in the supernatant after precipitation of yeast with 5-sulfosalicylic acid. The determined concentration in *S. cerevisiae* corresponded to $2{\cdot}5 \cdot 10^{-9}$ mol of SH groups per $5 \cdot 10^{-7}$ cells. Consequently, the low-molecular thiols form approximately only 5·5% of the content of total cell

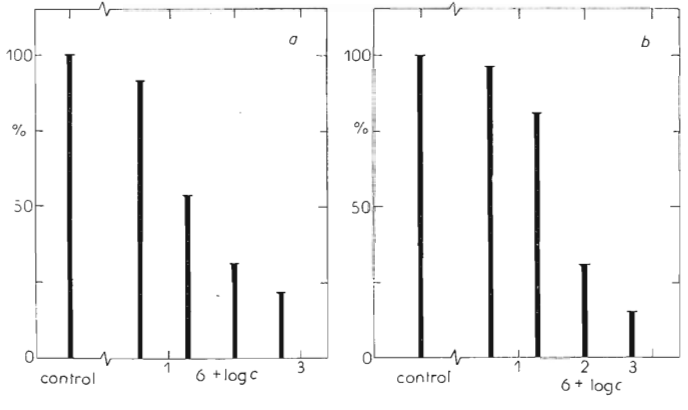

FIG. 3

The content of SH groups in *S. cerevisiae* XII (*a*) and *S. cerevisiae* XIIa (*b*) in the absence and in the presence of 1-(5-nitro-2-furyl)-2-nitroethylene. Content of SH groups in the yeast was determined after a 1 h-incubation with the inhibitor in a phosphate solution (0·1 mol/l) at 25°C. Concentration of the yeast was $5 \cdot 10^{7}$/ml. Furylethylene was applied in a final concentration $0{,}4 \cdot 10^{-6}$, $2 \cdot 10^{-5}$, $1 \cdot 10^{-4}$, and $5 \cdot 10^{-4}$ mol/l.

thiols when contrasted with thiolproteins. This means, at the same time, that the majority of modified SH groups is of a protein nature.

An analogous view on the reaction of furylethylenes with SH groups *in vivo* was obtained also with Ehrlich ascitic carcinoma cells. Table I summarizes results characterizing the effect of 1-(5-R-2-furyl)-2-nitro-2-methoxycarbonylethylenes upon the level of SH groups of EAC cells. Derivatives were applied in a uniform $1 \cdot 10^{-4}$ mol/l concentration into cells suspended in the Krebs–Ringer phosphate medium; the content of thiols was estimated after 30 and 120 min with Ellman reagent. The time-dependence aspects of effect of two furylethylenes investigated (R = H, R = $= COOCH_3$) is of interest. These lower the SH groups content after a 2 h-action less than after a 30 min-incubation. Such an increase of SH groups content after application of inhibitors is an already known phenomenon[17]; it is ascribed to the metabolism change, and therefore, to the content of SH containing compounds, as a defensive response of the biologic system toward the evoked inhibition. The modification degree of intracellular thiols by the respective derivatives is given by their reactivity towards thiols. The relationship between the titration degree of SH groups and the second order rate constants k ($l\,mol^{-1}\,s^{-1}$) characterizing the reactivity of the respective derivatives towards thioglycolic acid as a model thiol is given by equations (*1*) and (*2*). The first holds for a 30 min-incubation, the second for a 2 h-one:

$$\log(\% \text{ of free SH}) = -0{\cdot}321 \log k + 4{\cdot}096 \qquad (1)$$

$$\log(\% \text{ of free SH}) = -0{\cdot}392 \log k + 4{\cdot}572\,. \qquad (2)$$

TABLE I

Influencing the SH groups content of EAC cells with 1-(5-R-2-furyl)-2-nitro-2-methoxycarbonylethylenes. Concentrations of furylethylenes $1 \cdot 10^{-4}$ mol/l, of cells $1{\cdot}5 \cdot 10^6$/mol

Parameter	R				
	CH_3	H	Br	$COOCH_3$	NO_2
% SH after 30 min	99·8	94·5	79·2	58·4	46·0
% SH after 120 min	100·0	99·6	80·0	66·7	36·7
k, $l\,mol^{-1}\,s^{-1}$ [a]	$3{\cdot}3 \cdot 10^6$	$3{\cdot}9 \cdot 10^6$	$8{\cdot}2 \cdot 10^6$	$1{\cdot}5 \cdot 10^7$	$4{\cdot}0 \cdot 10^7$

[a] The second order rate constants ($l\,mol^{-1}\,s^{-1}$) determined for reactions of furylethylenes with thioglycolic acid as a model thiol were taken from[7].

TABLE II

ID_{50} Values determined graphically; molar concentrations of 1-(5-nitro-2-furyl)-2-R-ethylenes causing a 50% decrease of the SH groups content in EAC cells after a 2 h-incubation at 37°C

Parameter	R				
	NO_2	$COOC_2H_5$	$(NCH_3)_3Br$	CN	N_3
ID_{50}, mol/l^{-1}	$4{\cdot}8 \cdot 10^{-5}$	$9{\cdot}6 \cdot 10^{-5}$	$1{\cdot}0 \cdot 10^{-4}$	$1{\cdot}6 \cdot 10^{-4}$	$1{\cdot}6 \cdot 10^{-4}$
k, l mol^{-1} s^{-1} [a]	$5{\cdot}8 \cdot 10^{6}$	$8{\cdot}3 \cdot 10^{3}$	$2{\cdot}1 \cdot 10^{2}$	$9{\cdot}2 \cdot 10^{1}$	$5{\cdot}4 \cdot 10^{1}$

[a] The second order rate constants (l mol^{-1} s^{-1}) determined for reactions of furylethylenes with thioglycolic acid as a model thiol were taken from[7].

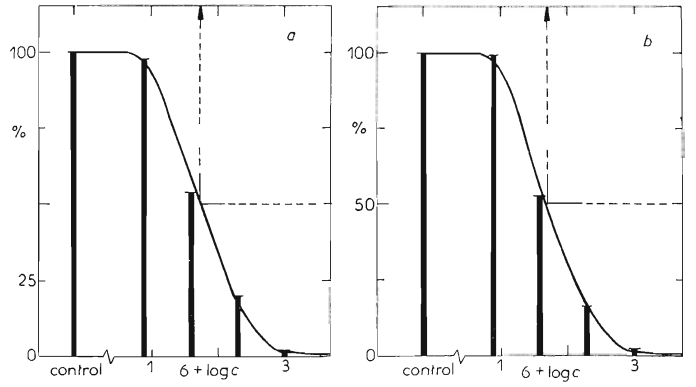

FIG. 4

Content of SH groups in the EAC cells in the absence and in the presence (*a*) of 1-(5-nitro-2-furyl)--2-nitro-2-methoxycarbonylethylene, and (*b*) 1-(5-nitro-2-furyl)-2-nitroethylene and the mode of ID_{50} value determinations (concentration of furylethylenes causing a 50% lowering of the level of thiols). The concentration of SH groups was determined (after a 2 h-incubation of EAC cells with furylethylenes in a Krebs–Ringer phosphate medium at 37°C) with Ellman reagent. Furylethylenes were applied in final concentrations $0{\cdot}8 \cdot 10^{-6}$, $4 \cdot 10^{-5}$, $2 \cdot 10^{-4}$, and $1 \cdot 10^{-3}$ mol/l. Concentration of cells $1{\cdot}5 \cdot 10^{6}$/ml. *a*: $ID_{50} = 5 \cdot 10^{-5}$ mol l^{-1}, *b*: $ID_{50} = 4{\cdot}8 \cdot 10^{-5}$ mol/l^{-1}

The correlation coefficient $r = -0.990$ and -0.977 for the first and second case respectively. The corresponding values of F tests are 103·9 and 64·9 ($n = 5$). As a consequence, a high reactivity towards thiols *in vivo* will be exhibited by those furylethylenes having the electron-accepting substituents at $C_{(5)}$ of the furan ring. Substituents of this type potentiate, however, the affinity of the electrophilic carbon of the exocyclic double bond of furylethylenes to SH groups[5].

Like relationship, describing the dependence of the reactivity of furylethylenes to thiols *in vitro* and *in vivo*, were also obtained with 1-(5-nitro-2-furyl)-2-R-ethylenes. Here, the degree of modification of intracellular thiols was characterized by the ID_{50} values, *i.e.* by concentrations of the appropriate derivatives causing a 50% decrease of the SH groups content with EAC cells after a 2 h-incubation. The graphic interpolation of the values for two tested derivatives shows Fig. 4. All data are listed in Table II. Also the reactivity of 1-(5-nitro-2-furyl)-2-R-ethylenes towards thiols, determined under the *in vitro* conditions, provides a basis for the concept on reactivity *in vivo*. This follows from equation (*3*):

$$\log (1/ID_{50}) = 0{\cdot}097 \log k + 3{\cdot}660 \qquad (3)$$

the correlation coefficient $r = 0{\cdot}950$ ($F = 28{\cdot}265$, $n = 5$). The ability to modify the SH groups of EAC cells of new derivatives of this series can be assesed on the basis of this equation.

Similarly, the low-molecular thiols in EAC cells represent a much smaller part of the total content of SH groups[17] (*c.* 19%; the over-all concentration of thiols as determined with Ellman reagent was $7{\cdot}5 \cdot 10^{-7}$ mol per $5 \cdot 10^{-7}$ cells). The prevalent part of SH groups modified with furylethylenes must be of a protein nature. It was really shown[18] that furylethylenes interact with thiol enzymes of EAC cells. Above all, the enzymes of glycolysis, especially hexokinase, phosphofructokinase and glyceraldehyde 3-phosphate dehydrogenase are involved. Reaction of furylethylenes with catalytically active SH groups of these enzymes leads to their inactivation. As a consequence, the slowing-down of glycolysis and subsequent biosynthetic processes, the stoppage of growth, and finally the dead of the cell[18] take place.

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