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Inhibitory Effect of Natural Phenolic Lipids upon NAD-Dependent Dehydrogenases and on Triglyceride Accumulation in 3T3-L1 Cells in Culture

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Alkylresorcinols are phenolic lipids present at levels of 0.03−0.15% in wheat and rye grains and almost 10 times higher in respective bran products. Despite numerous studies on the influence of dietary fibers on the regulation of energy metabolism, this issue still remains controversial. The objective of our current studies was to investigate whether 5-*n*-alk(en)ylresorcinols, natural phenolic components of high fiber human diets, may be considered as natural regulators of excessive fat accumulation. Our studies revealed that 5-*n*-alk(en)ylresorcinols isolated from wheat and rye bran inhibit glycerol-3-phosphate dehydrogenase, the key enzyme in triglyceride synthesis in adipocytes, specifically and effectively. Further in vitro studies showed that these compounds also prevent triglyceride accumulation in 3T3-L1 cells. The most effective compound in both systems was 5-*n*-heneicosylresorcinol. The results indicate that the potential to prevent triglyceride accumulation increases with the hydrophobicity of the phenolic inhibitor.

KEYWORDS: Phenolic lipids; resorcinolic lipids; alkylresorcinols; dehydrogenases; triglyceride accumulation; cereals; high fiber diet

INTRODUCTION

The assimilation, storage, and use of energy from nutrients constitute a homeostatic balance, which is essential to life. In vertebrates, the ability to store sufficient quantities of energy as triglycerides in adipose tissue can be maladaptive. Excessive fat accumulation brings along high risk to people suffering from pathological conditions such as hypertension, myocardial infarction, and diabetes (type II). On the other hand, epidemiological data link consumption of whole grain cereals to a decrease in the risk of many diseases observed in highly developed societies; for examples, see refs *1*−*3*.

The identification of effective inhibitors of triglyceride synthesis and/or storage may, therefore, provide one of several possible approaches to prevent undesired fat accumulation. One possible target in such efforts may be GPDH (EC 1.1.1.8), the key enzyme in phospholipid and triacylglycerol synthesis in adipose tissue (*4*). The activity of this enzyme increases several 100-fold during preadipocyte differentiation (*5*). Moreover, adipose tissue is the only human tissue lacking glycerol kinase activity (*6*). Thus, in this tissue, GPDH is practically the only source of glycerol-3-phosphate, an essential precursor in triglyceride synthesis.

Previously, Tsuge et al. (*7*) found that some alkylresorcinols (C15:0 and iso C15:0), produced by certain microorganisms, inhibit GPDH. The authors also showed that these compounds decrease triglyceride synthesis in adipocytes. Our data indicated that other natural 5-*n*-alk(en)ylresorcinols, isolated from wheat or rye bran, inhibit GPDH even more effectively (*8*). The fact that these resorcinols are natural components of a high fiber human diet (such as various bran-based products) suggests their possible participation in the prevention of excessive triglycerides accumulation in adipocytes. To establish any specificity of the inhibitory effect observed, in the present study, we examined the influence of resorcinolic lipids on other dehydrogenases (LDH, ADH, IDH, and Glu-6-PDH), thus elaborating our former studies. Furthermore, we determined the effect of these compounds on triglyceride accumulation in adipocytes.

MATERIALS AND METHODS

Materials. ADH (EC 1.1.1.1), GPDH (EC 1.1.1.8), IDH (EC 1.1.1.42), NADH, $NADP^+$, ATP, β-mercaptoethanol, HEPES, PBS, *p*-nitrophenyl phosphate, Triton X-100, triglyceride Sigma diagnostic kit, insulin, dexamethasone, MIX, and MTT were from Sigma Chemicals (Poznan, Poland); Glu-6-PDH (EC 1.1.1.49), hexokinase (EC 2.7.1.1), and LDH (EC 1.1.1.27) were from Boehringer (Mannheim, Germany); HBSS, DMEM, trypsin, glutamine, and antibiotics were from Gibco BRL (Vienna, Austria); EDTA was from Serva (Heidelberg, Germany); and RP-TLC 18 Plates were from Merck (Warsaw, Poland). All other reagents were of the highest available purity.

All resorcinolic lipid homologues used in these studies were isolated from rye and wheat bran acetone extracts using the chromatographic method described earlier (*9*). In our studies, we have focused on the homologues that are most abundant in most cereal materials (rye, wheat,

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triticale, and barley), i.e., C15−C21 alk(en)ylresorcinols. A similar chromatographic procedure was used to isolate phenols from extracts obtained from *Anacardium occidentale* nuts (technical CNSL and natural CNSL). The purity of obtained preparations was above 99% as revealed by the gas chromatography−mass spectrometry (GC-MS) analyses (*10*). The concentration of resorcinolic lipids in the stock solutions was determined by the microcolorimetric method of Tłuścik et al. (*11*).

Cardol, a natural mixture of unsaturated (mostly trienoic) C15 alkylresorcinol congeners from CNSL, an oily extract obtained during the thermal processing of the cashew nuts; cardanol, a natural mixture of unsaturated C15 alkylphenol congeners from CNSL; and anacardic acid, a natural mixture of unsaturated C15 alkylphenolic acid congeners from the natural CNSL [the extract from cashew nuts that was not subjected to the thermal processing (for details, see refs *12* and *13*)] were used.

Methods. *Enzymes Assays.* A fixed volume (100 μL) of methanolic solutions of resorcinolic lipids was added to the reaction mixture. The reaction was initiated by adding 150 μL of each enzyme solution (2 U/mL). The changes in absorbance at 340 nm were monitored in a Varian Inc. model CARY-1E spectrophotometer for 15 min at room temperature (21−23 °C).

GPDH activity was assayed by the method of Wise and Green (*14*). The standard reaction mixture (final volume of 3 mL) consisted of 100 mM triethanolamine-HCl buffer, pH 7.5, 2.5 mM EDTA, 0.12 mM NADH, 0.6 mM dihydroxyacetone phosphate, and 0.1 mM β-mercaptoethanol, relative to all of the particular components.

The activity of ADH was assayed by the modified method of Anderson and Reynolds (*15*). The standard reaction mixture (final volume of 3 mL) consisted of 10 mM pyrophosphate buffer, pH 7.95, 7 mM acetaldehyde, and 0.12 mM NADH, relative to all of the components.

The activity of IDH was assayed by the method of Eguchi et al. (*16*). The standard reaction mixture (final volume of 3 mL) consisted of 50 mM HEPES−Na buffer, pH 7.9, 0.5 mM $MnCl_2 \cdot 4H_2O$, 0.57 mM isocitrate, and 0.12 mM NAD^+, relative to all of the components.

The activity of LDH was assayed by the method of Dabrowska and Gutowicz (*17*). The standard reaction mixture (final volume of 3 mL) consisted of 100 mM phosphate buffer, pH 7.5, 10 mM pyruvate, and 0.12 mM NADH, relative to all of the components.

The activity of Glu-6-PDH was assayed by the method of Beutler (*18*) with the following modifications. The standard reaction mixture (final volume of 3 mL) consisted of (final concentrations in the reaction mixture) 100 mM imidazole, pH 6.9, 5 mM $MgCl_2$, 0.12 mM $NADP^+$, 1 mM ATP, and 1 mM glucose. The reaction was initiated by adding 150 μL of hexokinase solution (2 U/mL). After the time necessary for the complete conversion of glucose to glucose-6-phosphate, established experimentally, 150 μL of Glu-6-PDH solution (2 U/mL) was added to the reaction mixture. A fixed volume (100 μL) of resorcinolic lipids was added at that stage. The changes in absorbance at 340 nm were monitored by a Varian Inc. model CARY-1E spectrophotometer for 15 min at room temperature (21−23 °C).

Cell Culture and 3T3-L1 Cells Conversion to Adipocytes. Cultivation of 3T3-L1 cells and their conversion to adipocytes was carried out according to the method of Aratani and Kitagawa (*19*) with the following modifications. The medium used in all experiments was DMEM, which contained 10% FCS, glutamine (59 μg/mL), and antibiotics [penicillin (0.1 mg/mL) and streptomycin (0.1 mg/mL)]. The induction medium additionally contained 0.25 μM dexamethasone, 0.5 mM MIX, and 10 μg/mL insulin. After induction for 2 days, the medium was replaced with DMEM medium containing 10% FCS and the appropriate concentration of resorcinolic or phenolic lipids. The cells were cultivated under those conditions for 7 days. After this period, the amount of accumulated triglycerides was assayed. The stock solutions of MIX and phenolic and resorcinolic lipids were prepared in DMSO.

Triglyceride Assay. Cultured cells were detached from the plate with trypsin and centrifuged. Subsequently, they were resuspended in Tris−HCl buffer (pH 7.5) containing EDTA (0.416 mg/mL) and then sonicated. The triglyceride content was determined using triglycerides Sigma diagnostic kit according to the manufacturer's instructions.

Cytotoxicity Assay. The cytotoxicity of resorcinolic and phenolic lipids used in these studies was determined on 3T3-L1 cells using the MTT assay (*20*). The test is based on the detection of cellular mitochondrial activity. Briefly, the cells were incubated with studied lipids for 7 days. Then, the surviving fraction was estimated by the MTT dye assay as follows: 20 μL of 5 mg/mL MTT was added to each well, and incubation was continued for a further 4 h. Then, the medium was carefully removed from each well. Subsequently, 100 μL of DMSO was added into each well and the solution was agitated to give a uniform color that was read at 520 nm.

Statistics. All experiments were done at least in triplicate, and the data were analyzed statistically. The standard errors did not exceed 5% in any determinations. Statistical significance of differences was evaluated by a two-tailed unpaired Student's *t*-test using the in-build feature of the SlideWrite software (Advanced Graphics Software, Inc.).

RESULTS AND DISCUSSION

Effect of 5-*n*-Pentadecylresorcinol on the Activity of ADH, LDH, IDH, and Glu-6-PDH. In our former studies, we have demonstrated that resorcinolic lipids compete with NADH for the coenzyme binding site (*8*). Hence, we considered it also possible that other NAD-dependent dehydrogenases could be inhibited by these compounds. Therefore, we selected four representative specimens, all acting on the CH−OH group of the donor, with NAD^+ or $NADP^+$ as acceptors: ADH (EC 1.1.1.1), Glu-6-PDH (EC 1.1.1.49), IDH (EC 1.1.1.42), and LDH (EC 1.1.1.27). These enzymes were selected on one of two criteria: the similarity of their coenzyme binding domains (*21*) (ADH, LDH) or the literature data on their inhibition by compounds belonging to the phenolic lipids (*22*) (IDH, Glu-6-PDH).

An additional argument to choose ADH was that this enzyme, like GPDH, is inhibited by N^1-alkylnicotinamide chlorides (*23*). The specific binding of these structural analogues of NADH is not a general property of dehydrogenases.

As a model resorcinolic lipid [alk(en)ylresorcinol], we used 5-*n*-pentadecylresorcinol in all experiments. The standard incubation mixtures contained increasing amounts of 5-*n*-pentadecylresorcinol, up to 50 μM, which was approximately 1 order of magnitude higher than the IC_{50} value estimated previously for GPDH (*7*, *8*).

Table 1 demonstrates that ADH, Glu-6-PDH, IDH, and LDH show ≤5% inhibition by 5-*n*-pentadecylresorcinol at a concentration of 20 μM, while earlier (*8*) GPDH was shown to be inhibited by 50% already at 4.1 μM and even by 100% at 11.5 μM.

LDH was inhibited by 5-*n*-pentadecylresorcinol by only 30%. The activity of this enzyme was not affected at all by any of the compounds tested at concentrations at which they fully inhibited GPDH (11.5 μM) (*8*). The feature by which these two enzymes significantly differ is the flexible loop (αD) of the LDH (*24*). The analysis of this particular fragment of LDH reveals that five of the 10 amino acids engaged in the coenzyme binding occur within this loop or in its close proximity (*24*).

The loop itself is very flexible, and the coenzyme binds first to it. The interaction between the coenzyme and the protein results in the correct conformation needed for further catalysis (*25*). The binding of the nucleotide causes substantial structural changes that apparently enhance the enzyme's ability to bind substrates. This region seems to be very important for the enzyme function, since its amino acid composition is almost identical in all known LDHs isolated from different species and tissues (*21*, *26*). It seems very likely that this loop is also responsible for the phenomenon that alkylresorcinols inhibit LDH not nearly as effectively as they inhibit GPDH. Only

Table 1. Effect of 5-*n*-Pentadecylresorcinol on the Activity of ADH, LDH, IDH, GPDH, and Glu-6-PDH[a]

	inhibition of the enzyme (%) corresponding to different concentrations (μM) of 5-*n*-pentadecylresorcinol									
	4	11	15	20	25	30	35	40	45	50
GPDH	50 ± 5	100 ± 2								
ADH	0	0	0	5 ± 2	8 ± 4	15 ± 3	25 ± 2	28 ± 3	30 ± 2	30 ± 2
LDH	0	0	0	5 ± 1	11 ± 2	15 ± 3	20 ± 2	25 ± 4	28 ± 3	30 ± 2
G-6-PDH	0	0	0	0	7 ± 1	12 ± 3	15 ± 2	18 ± 3	20 ± 2	20 ± 2
IDH	0	0	0	0	0	0	0	0	0	0

[a] The experimental conditions of the assays are described in the Materials and Methods. The data are the means ± standard deviation of triplicate samples from two separate experiments.

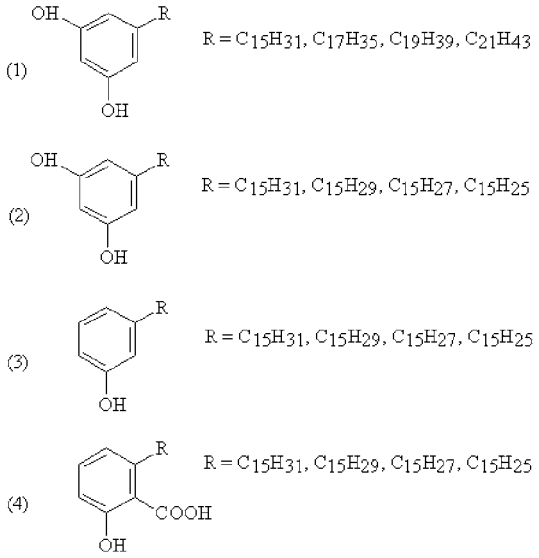

Figure 1. Chemical structures of resorcinolic and phenolic lipids used in these studies. (1) 5-*n*-Alkylresorcinols, homologues isolated from cereal grains. (2) Cardols, natural mixture of C15 alkylresorcinol congeners from CNSL (technical). In fact, according to the literature, there are several cardols—the "original one" from *Anacardium* that is a mixture of 15:0, 15:1, 15:2, and 15:3 congeners (*12*)—but there are also cardols 17:1 and C11 as well as C13 cardols. This name was ascribed on the basis of the ring structure. All 1.3 dihydroxyalk(en)ylbenzenes are cardols (or cardol homologues); see ref *13*. (3) Cardanols, natural mixture of C15 alkylphenols from CNSL (technical). (4) Anacardic acids, natural mixture of C15 alkylphenolic acids from CNSL (natural).

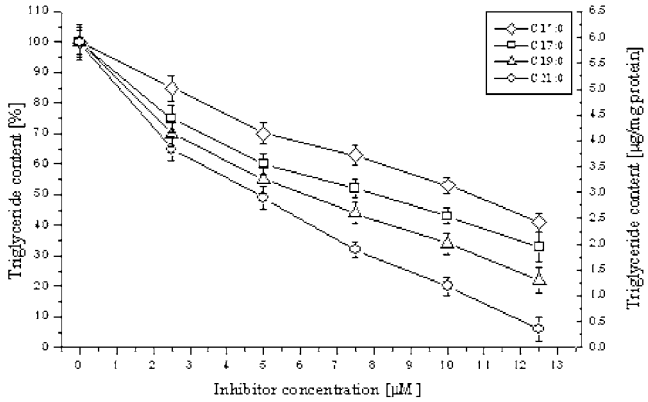

Figure 2. Effect of alkylresorcinol homologues on the triglyceride content of 3T3-L1 cells. 3T3-L1 cells were converted to adipocytes according to the procedure described in the Materials and Methods. After it was induced for 2 days, the medium was replaced with DMEM containing 10% FCS and resorcinolic lipids prepared in DMSO. The cells were cultivated under those conditions for 7 days. After this period, the amount of accumulated triglycerides was assayed. Pentadecylresorcinol (◇), heptadecylresorcinol (□), nonadecylresorcinol (△), and heneicosylresorcinol (○). Mean values ± SD were obtained from three separate experiments carried out in duplicate.

NADH, but not resorcinolic lipids, can induce these conformational changes in the coenzyme binding domain, which is necessary for catalytic activity.

ADH was inhibited by 5-*n*-pentadecylresorcinol not more than 30% (**Table 1**). What differences in the topology of the two enzymes may be responsible for their different sensitivity to alkylresorcinols? One of the most important is that ADH has a Zn^{2+} ion at its active site and its enzymatic mechanism appears to be based on electrostatic effects of the metal ion rather than on general acid- or base-mediated catalysis typical for LDH and GPDH.

We found that IDH was the only enzyme that was not inhibited by 5-*n*-pentadecylresorcinol at all. This is in contradiction with the data obtained by Vincieri et al. (*22*) who demonstrated that IDH was inhibited by phenolic lipids extracted from *Gingko biloba*.

Effect of Alk(en)y Resorcinols on the Triglyceride Content of 3T3-L1 Cells. In our previous studies, we demonstrated that phenolic lipids, particularly resorcinolic lipids, were effective and specific GPDH inhibitors (*8*). Thus, we considered it of interest to examine whether these compounds also affect triglyceride accumulation in 3T3-L1 cells. In subsequent experiments, we tested several representatives of phenolic lipids, namely, 5-*n*-pentadecylresorcinol (C 15:0), 5-*n*-heptadecylresorcinol (C 17:0), 5-*n*-nonadecylresorcinol (C 19:0), 5-*n*-heneicosylresorcinol (C 21:0), cardol, cardanol, and anacardic acid (for chemical formulas, see **Figure 1**). The first four compounds are the most frequently occurring homologues in all cereal grains and whole grain/bran products; see, for example, refs *27* and *28*.

To examine whether the length of the alkylresorcinol side chain can affect the process of triglyceride accumulation in adipocytes, we analyzed the inhibitory activities of four resorcinolic lipid (alkylresorcinol) homologues (**Figure 2**). The results indicate that the length of the alkyl side chain influences the inhibitory potential of these compounds on triglyceride storage. 5-*n*-Heneicosylresorcinol (C 21:0), the homologue with the longest side chain studied, reduced the amount of accumulated triglycerides most effectively (**Table 2**). This compound, at the highest concentration used (12.5 μM), reduced the amount of stored fat by more than 90%. The least efficient was the 5-*n*-pentadecylresorcinol (C 15:0) that decreased the amount of stored triglycerides only by 60%. It can be concluded that the length of the aliphatic side chain is one of the factors that determine the inhibitory potential on triglycerides accumulated in 3T3-L1 cells. Moreover, the comparison of the data obtained for 5-*n*-pentadecylresorcinol and cardol (**Figures 2** and **3**) indicates that unsaturation of the side chain also

Table 2. IC_{50} Values (the Concentration of the Inhibitor that Reduced the Amount of Triglycerides by 50%) for Various Resorcinolic Lipid Homologues[a]

alkylesorcinol	IC_{50} (μM) ± SD
5-*n*-pentadecylresorcinol (C 15:0)	10.7 ± 0.2
5-*n*-heptadecylresorcinol (C 17:0)	8.2 ± 0.3
5-*n*-nonadecylresorcinol (C 19:0)	6.3 ± 0.2
5-*n*-heneicosylresorcinol (C 21:0)	5.0 ± 0.1

[a] After 2 days of conversion, 3T3-L1 cells were further cultivated in the presence of alkylresorcinols at various concentrations for 7 days. After that period, the amount of accumulated triglycerides was assayed using triglycerides Sigma diagnostic kit. The data are the means ± standard deviation of triplicate samples from two separate experiments. The difference between the IC_{50} values for the different alkylresorcinol homologues was statistically significant, as evaluated by a two-tailored Student's *t*-test.

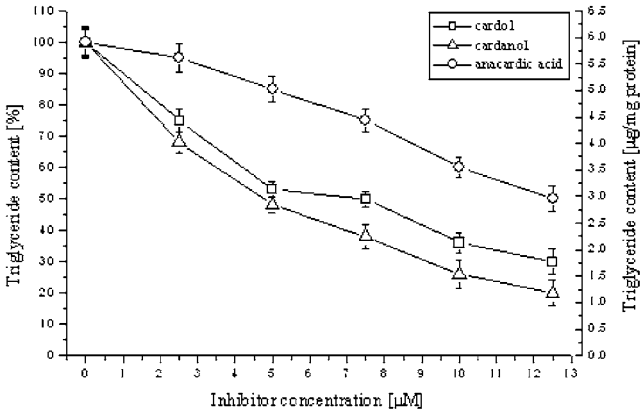

Figure 3. Effect of cardanol (△, natural mixture of unsaturated C15 alkylphenol congeners from technical CNSL), cardol (□, natural mixture of unsaturated C15 alkylresorcinol congeners from technical and natural CNSL), and anacardic acid (◇, natural mixture of unsaturated C15 alkylphenolic acid congeners from natural CNSL) on the triglyceride content of 3T3-L1 cells. The experimental conditions were as described in **Figure 2**. For the explanation of the terms technical and natural CNSL, see refs *12* and *13*.

enhances the potential to decrease the amount of stored triglycerides.

We previously demonstrated (*8*) that the number of hydroxyl groups in the aromatic ring of the inhibitor is also an important determinant of its GPDH inhibiting potency. Thus, we verified whether this parameter also affects fat storage in 3T3-L1 cells. For these experiments, we compared the effect of cardol (dihydroxy phenolic compound), cardanol (monohydroxy phenolic compound), and anacardic acid (alkylphenolic acid), compounds of identical side chain unsaturation, on fat accumulation in 3T3-L1 cells.

The data, presented in **Figure 3**, indicate that all phenolic lipids studied induced a substantial decrease (by 50−70%) of triglyceride accumulation. Cardanol was found to be the most potent inhibitor, although cardol was only slightly less effective. Comparing the data obtained for anacardic acid and cardol, we observe that the presence of a carboxyl group, which at physiological pH is ionized, resulted in a decrease of the inhibitory potential of this compound. The difference in inhibitory abilities is even more pronounced when the results for cardanol and anacardic acid are directly compared.

Our data demonstrate that the larger the hydrophobic moiety of a phenolic lipid, the higher its potential to inhibit triglyceride accumulation in adipocytes. Correspondingly, it has been demonstrated that the larger the hydrophobic moiety of a

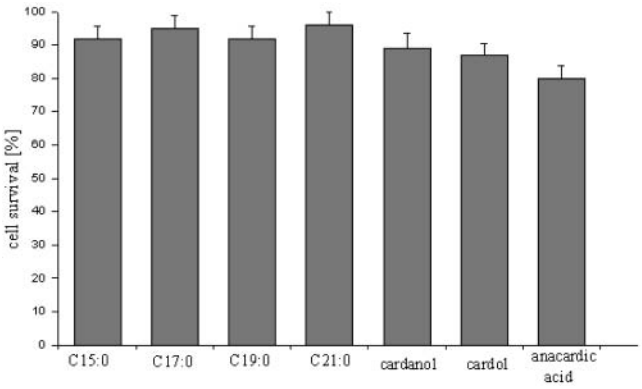

Figure 4. Cytotoxicity of resorcinolic and phenolic lipids determined on 3T3-L1 cells. The cells were incubated with lipids for 7 days. The surviving fraction was determined by the MTT dye assay described in the Materials and Methods. The data are expressed as the percentage of surviving cells, relative to control 3T3-L1 cells (100%). Mean values ± SD were obtained from three separate experiments carried out in duplicate.

phenolic lipid, the easier it incorporates into membranes and the faster it diffuses across the membrane (*29*). Therefore, we conclude that the potential of a phenolic lipid to inhibit triglyceride accumulation depends on its ability to incorporate into biological bilayers and on its flexibility in this environment.

Because our previous studies showed that GPDH is effectively inhibited by resorcinolic lipids (*8*), we suggest that the target of their action in 3T3-L1 cells is also this enzyme. Unpublished studies of our group showed that GPDH interacts with phospholipid bilayers. Such an interaction may significantly modulate the enzyme activity. This kind of interaction is considered to be one of several possible ways of the regulation of enzyme activity (*30*−*32*) and could also be involved in the case of GPDH.

To determine whether phenolic lipids may have any direct cytotoxic effect [as was suggested earlier by other authors and discussed in our previous article (*33*)] on 3T3-L1 cells, we incubated the cells in the presence of each individual inhibitor studied, at a concentration of 12.5 μM. After 7 days, the number of surviving 3T3-L1 cells was determined. For all compounds used, the number of surviving cells was practically identical to that of the control experiment (differences were statistically insignificant) (**Figure 4**). Thus, it can be concluded that resorcinolic and phenolic lipids do not have any direct cytotoxic effect on 3T3-L1 cells in culture, even at the highest concentration used in the triglyceride accumulation experiments (12.5 μM).

In conclusion, the experiments presented in this paper are important from the viewpoint that resorcinolic lipids may have potential as drugs acting against excessive triglyceride accumulation. They also throw more light on the role of wheat and rye bran products present in human daily diet and contribute to the understanding of the molecular mechanism by which its individual constituents may influence human metabolism. Although Plato already (5th−4th century BC) advised his compatriots to consume meals rich in cereals, the mechanism by which whole grain diet exerts its beneficial effects is still elusive.

Our current experiments focus on the question of whether resorcinolic lipids are able to inhibit fat storage under in vivo conditions. Previous data obtained by Nakatsu and colleagues make us believe that these compounds will also reduce fat deposits in experimental animals. Yet, their results show that animals receiving anacardic acid in their diet lost approximately

10% of the initial body weight (*34*). Recent data indicate that cereal grain alkylresorcinols are also absorbed from the diet in animals and humans and that they decrease liver cholesterol in rats (*28*).

ABBREVIATIONS USED

ADH, alcohol dehydrogenase; CNSL, cashew nut shell liquid; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; GPDH, glycerol-3-phosphate dehydrogenase; Glu-6-PDH, glucose-6-phosphate dehydrogenase; HBSS, Hank's balanced salt solution; IDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MIX, 1-methyl-3-isobutylxanthine; PBS, phosphate-buffered saline; MTT, 3(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; HEPES, 4-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); RP-TLC, reversed phase thin-layer chromatography.

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