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Oxidation of carbidopa by tyrosinase and its effect on murine melanoma

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ABSTRACT

Oxidation of the anti-Parkinsonian agent carbidopa by tyrosinase was investigated. The products of this reaction were identified as 3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid and 6,7-dihydroxy-3-methylcinnoline. These results demonstrate that after oxidation of the catechol moiety to an *o*-quinone either a redox exchange with the hydrazine group or a cyclization reaction occur. The cyclization product underwent additional oxidation reactions leading to aromatization. The cyclization reaction is undesired in the case of hydrazine-containing anti-melanoma prodrugs and will have to be taken into account in designing such compounds. Carbidopa was tested against B16(F10) melanoma cells in culture and showed cytotoxicity significantly higher than either of its oxidation products and L-dopa. This effect, however, was not specific to this cell line.

Carbidopa ((2*S*)-3-(3,4-dihydroxyphenyl)-2-hydrazinyl-2-methylpropanoic acid) is an aromatic amino acid decarboxylase inhibitor[1] which was introduced as a co-drug with L-dopa in the treatment of Parkinson's disease in the early 1970s.[2–4] Its metabolism was studied primarily in vivo and several metabolites were identified in plasma and urine of a number of mammalian species, including humans, in free form or as glucuronate conjugates: 3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid, 3-(4-hydroxy-3-methoxyphenyl)-2-methylpropanoic acid, 3-(3-hydroxyphenyl)-2-methylpropanoic acid, 3-(4-hydroxy-3-methoxyphenyl)-2-methyllactic acid, 3-(3-hydroxyphenyl)-2-methyllactic acid, and 3,4-dihydroxyphenylacetone.[5–7] These products suggest participation of oxidases, catechol *O*-methyltransferase and dehydroxylating systems in the metabolism of this drug. However, involvement of any particular enzyme in the degradation of carbidopa has not been studied so far. Sweet potato polyphenol oxidase was applied for flow injection-spectrophotometric determination of L-dopa and carbidopa in pharmaceutical formulations,[8] but no attempts were made to identify the products of the oxidation of carbidopa by this enzyme. It was even speculated that the product of this reaction was dopachrome, although the UV–vis spectrum of the chromophore differed markedly from dopachrome, which cannot be formed in this reaction. The spectrum obtained after oxidation of carbidopa by polyphenol oxidase shows two maxima: at ca. 360 and 410 nm[8] and cannot be attributed to any of the metabolites identified so far, which do not absorb in this region.

We have recently shown that the hydrazine group in amino acid phenylhydrazides[9] and in the antitumor drug procarbazine[10] can be oxidized by *o*-quinones and therefore indirectly by the action of tyrosinase. Based on these results we have postulated that this redox exchange reaction can be utilized in activation of anti-melanoma prodrugs with a hydrazine linker. Carbidopa, an approved drug containing both the catechol and hydrazine moieties was an obvious choice to test the reaction in an intramolecular setting before designing target compounds.

We first performed the reaction of 0.1 mM carbidopa (Sochinaz S.A., Vionnaz, Switzerland) with mushroom tyrosinase[11] (specific activity 2956 U/mg) and monitored it spectrophotometrically. The reaction was carried out in 2.6 mL of 100 mM sodium phosphate buffer pH 6.8. We observed a spectrum very similar to that obtained in the reaction catalyzed by sweet potato polyphenol oxidase[8] with a maximum at 370 nm (Fig. 1). The second maximum at 410 nm was transient and remained for prolonged periods of time only if the substrate concentration was 0.25 mM or higher (data not shown). We next carried out oxygen consumption measurements at 0.1 mM concentration of substrates (12 mL of 100 mM sodium phosphate buffer, pH 6.8), which demonstrated that the reaction rate for carbidopa was slightly lower than for L-dopa (0.19 ± 0.030 μM/s and 0.27 ± 0.027 μM/s, respectively) and less oxygen was consumed per mole of carbidopa (80 μM and 91 μM after 60 min incubation for dopa and carbidopa, respectively, Fig. 2). The reaction was then performed at 0.25 mM or 0.4 mM concentration of carbidopa, under conditions where oxygen was the limiting agent, to prevent secondary oxidations and polymerization, and the mixture was analyzed by HPLC (Altech Alltima

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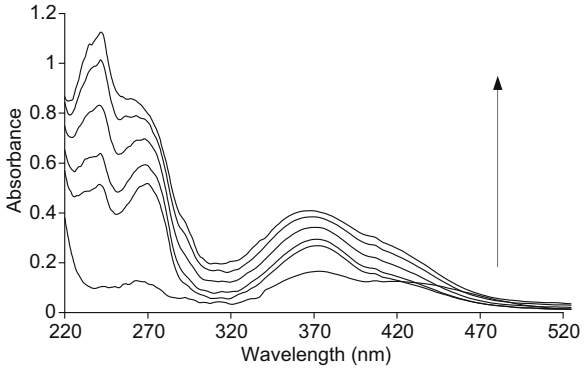


Figure 1. Spectral changes during oxidation of 0.1 mM carbidopa by 5 μg of tyrosinase in 2.6 mL of 100 mM sodium phosphate buffer, pH 6.8. The spectra displayed were recorded immediately after addition of the enzyme and then after 2, 4, 6, 10, 20, and 30 min. The arrow shows the direction of spectral changes (increase of absorbance).

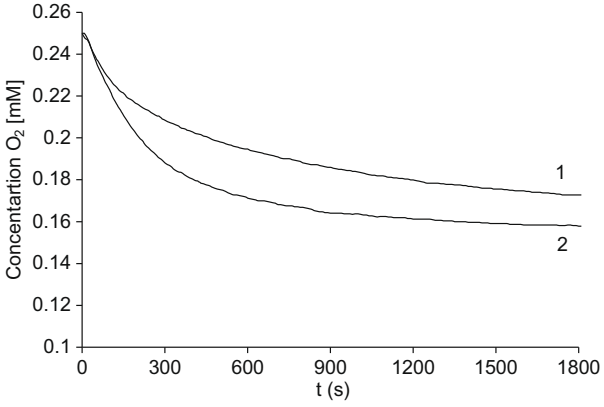


Figure 2. Oxygen consumption measurements during oxidation of 0.1 mM carbidopa (1) and 0.1 mM L-dopa (2) by 46 μg of tyrosinase in 12 mL of 100 mM sodium phosphate buffer, pH 6.8.

C_{18}, 150 × 3 mm column connected to a Beckman System Gold instrument with a diode array detector and a 20 μL sample loop). Separation was performed with 0.1% TFA in water and acetonitrile as the mobile phase (10% acetonitrile for 2 min, then 10–60% acetonitrile gradient in 18 min), at a flow rate of 0.4 mL/min. Chromatograms were recorded at 220, 280, and 340 nm. Two products were detected in the reaction mixture in addition to the unreacted substrate (Fig. 3). To identify them, a preparative reaction was performed at 45 mg scale (0.25 mM carbidopa concentration, 800 mL of 10 mM sodium phosphate buffer, pH 6.8). After the UV–vis spectra remained unchanged, the enzyme was removed from the reaction mixture by ultrafiltration (Amicon Ultra-15, 10,000 MWCO, Millipore), the filtrate was concentrated by evaporation to ca. 1/10 of the initial volume and loaded on a 16 mL C_{18} column (Bakerbond Octadecyl). Elution was performed with a stepwise gradient of acetonitrile in water. Fractions were analyzed spectrophotometrically and by TLC. Three products were detected, isolated and identified. The major product with a retention time of 11.5 min in HPLC analysis was identified by NMR analysis as 3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid (**1**, Scheme 1): ^{1}H (CD_3OD, Bruker Ultrashield 400 MHz)—1.065 (3H, d), 2.433 (1H, dd), 2.549 (1H, m), 2.840 (1H, dd), 6.516 (1H, dd), 6.624, (1H, d), 6.640 (1H, d). The spectrum of this product showed the characteristic signals of nonequivalent methylene protons also present in the substrate (2.433 and 2.840 ppm for this compound, 2.675 and 2.784 ppm for carbidopa). The second product with a retention

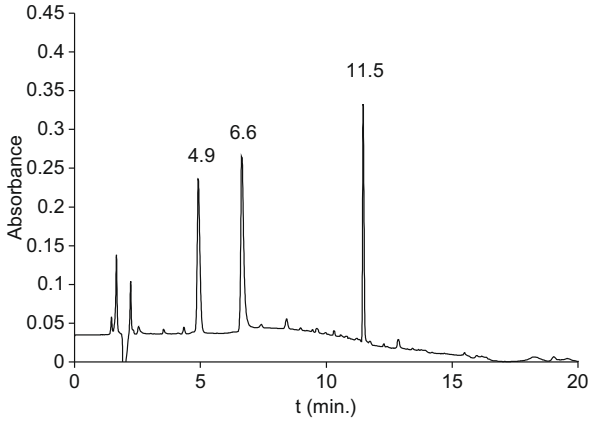
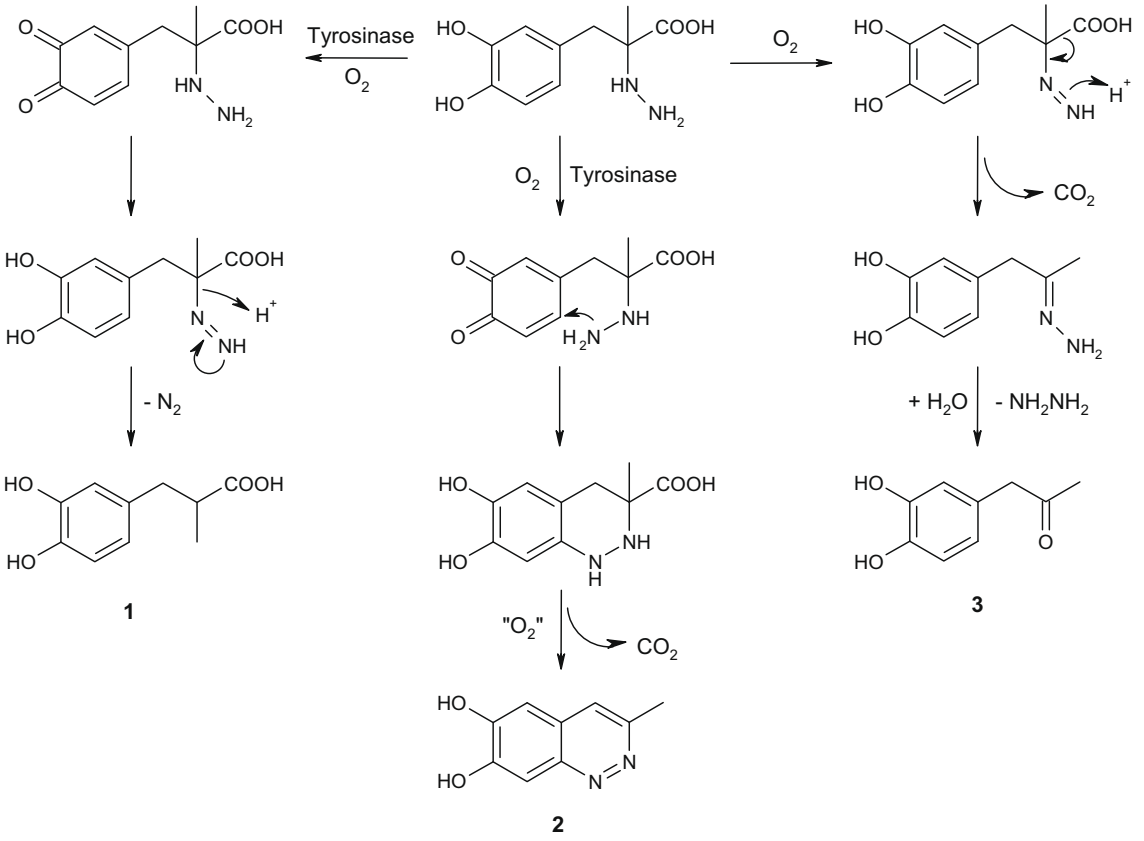


Figure 3. Chromatographic analysis of a mixture of 0.25 mM carbidopa with 5 μg of tyrosinase in 1 mL of 100 mM sodium phosphate buffer, pH 6.8, after 30 min incubation (detection at 220 nm). Retention times determined by analysis of standards were: 4.9 min for carbidopa, 6.6 min for 6,7-dihydroxy-3-methylcinnoline, 11.5 min for 3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid, and 16.4 min for 3,4-dihydroxyphenylacetone (not seen in the presented chromatogram).

time of 6.6 min in HPLC analysis gave an NMR spectrum characteristic for a bicyclic aromatic compound: ^{1}H (D_2O)—2.625 (3H, s), 6.815 (1H, s), 7,298 (1H, s), 7.549 (1H, s); ^{13}C (D_2O)—20.43, 103.67, 105,99, 117,93, 125,43, 145.82, 148.70, 153.36, 157.55). The structure was determined from an ^{1}H, ^{13}C HMBC spectrum and LC/ESI-MS analysis (Bruker MicrOTOF-Q, 177.1 a.m.u. in a positive ion mode, 175.1 a.m.u. in a negative ion mode) as 6,7-dihydroxy-3-methylcinnoline (**2**, Scheme 1). The UV–vis spectrum of the isolated compound was very similar to that of the reaction mixture (Fig. 4). These results demonstrate that after oxidation of the catechol moiety to an *o*-quinone by tyrosinase either the redox exchange (intramolecular or intermolecular) with the hydrazine group or the nucleophilic attack of the latter on the former take place. Possible pathways leading to the formation of these two products are presented in Scheme 1. The mechanisms proposed are consistent with the results of in vivo studies of the metabolism of carbidopa, which did not detect hydrazine in the urine or plasma of experimental animals.[5,6] The pathway leading to the formation of 3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid, postulated by the authors, included oxidation of the hydrazine group and its loss as a nitrogen molecule.[6] 6,7-Dihydroxy-3-methylcinnoline is formed by cyclization of the *o*-quinone (nucleophilic attack of the hydrazine nitrogen atom) and a subsequent 4-electron oxidation. However, steps and factors participating in the formation of this bicyclic aromatic product remain unclear.

The cyclization of *o*-quinones with a hydrazine group in the side-chain is an undesired side-reaction from the point of view of designing anti-melanoma prodrugs.[10] It may reduce the yield of effector release in the case of dialkyl hydrazines. Cyclization should not occur, however, in the case of hydrazides, carbazates or semicarbazides. Acylation of the hydrazine moiety at the distal nitrogen atom should make it insufficiently nucleophilic, as it has been recently demonstrated for dopamine derivatives.[12]

Small amounts of 3,4-dihydroxyphenylacetone were also isolated from our reaction mixtures: ^{1}H (DMSO-d_6)—2.047 (3H, s), 3.502 (2H, s), 6.428 (1H, dd), 6.557 (1H, d), 6.651 (1H, d) (**3**, Scheme 1). This compound was not detected in the HPLC analysis of the original reaction mixtures (retention time of a synthetic reference was 16.4 min), which confirms previous suggestions that it is produced from carbidopa during sample manipulation.[6] 3,4-Dihydroxyphenylacetone was the major product obtained previously after electrochemical and chemical oxidation of carbidopa.[13] Two oxidation reactions were detected by cyclic voltammetry and



Scheme 1. Postulated reactions occurring after oxidation of carbidopa by tyrosinase: *o*-quinone may undergo a redox exchange reaction with the hydrazine group leading to the azo derivative and regenerating the catechol; loss of a nitrogen molecule gives 3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid (**1**); nucleophilic attack of the hydrazine distal nitrogen atom on the *o*-quinone generates a bicyclic product, whose oxidation leads to 6,7-dihydroxy-3-methylcinnoline (**2**); non-enzymatic oxidation of the hydrazine group followed by decarboxylation leads to a hydrazone, which upon hydrolysis gives 3,4-dihydroxyphenylacetone (**3**).

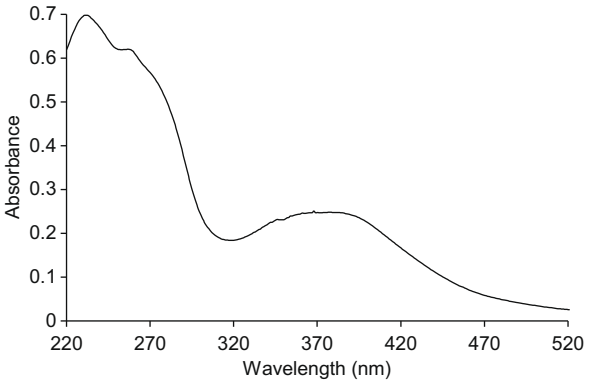


Figure 4. The UV–vis spectrum of the isolated 6,7-dihydroxy-3-methylcinnoline.

differential-pulse voltammetry. One of them was assigned to the catechol group and the other one to the carboxylate. Carbon dioxide and hydrazine were detected as side products.[13] However, the postulated pathway leading to 3,4-dihydroxyphenylacetone included the formation of a carbocation connected to a hydrazine group[13], which seems extremely unlikely. We believe that the second oxidation process with E_p = +980 mV in differential-pulse voltammetry corresponds to the oxidation of the hydrazine group and not the carboxylate, as proposed by the authors,[13] and the reactions proceed by a pathway presented in Scheme 1.

Recently, the cytotoxic effect of carbidopa has been demonstrated against human pulmonary carcinoid and small cell lung carcinoma cells,[14] which was later shown to be due to hydrogen peroxide generated by the autooxidation of this compound.[15] The generation of H_2O_2 was attributed to the catechol moiety, whereas the hydrazine group was not considered in this process. A mixture of L-dopa and carbidopa was also applied in earlier studies of the anti-melanoma effect of 4-*S*-cysteaminylphenol and its *N*-acetyl derivative.[16] This mixture significantly improved the cytotoxicity of the tested compounds. A combination of L-dopa and carbidopa was also tested in patients with metastatic malignant melanoma.[17] However, it was ineffective even at the highest tolerated doses and severe side effects were observed, which included gastrointestinal toxicity and postural hypotension. Levodopa–carbidopa mixture also inhibited the transformation of lymphocytes derived from patients with malignant melanoma.[18] We have, however, not found any reports describing the effect of carbidopa alone against melanoma. We have therefore tested it in cell cultures and compared the effect with that of dopa.

We used B16(F10) murine melanoma cells (from the American Type Culture Collection, ATCC) and two control cell lines: NIH3T3 (ATCC) and HECa10 (lymph node-derived and provided by Dr. D. Duś, Institute of Immunology and Experimental Therapy, Wrocław, Poland). Cells (2×10^3 cells per well) were cultured in RMPI 1640 medium (100 μL) supplemented with 10% FBS using NUNCLON™ Surface (NUNC™) 96-well plates. Cultures were kept in a humidified standard incubator (37 °C and 5% CO_2). After 24 h, the tested compounds were added in quadruplicate to the culture media at eleven different concentrations (10–2000 μM). The culture vessels were incubated for further 24 or 48 h, the culture media were replaced with MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.5 mg MTT/1 mL PBS) and the cells

were incubated for 3 h at 37 °C. The formed formazan crystals were dissolved in acid–isopropanol solution. Spectrophotometric measurements were performed using ELISA EL_x800 reader (Bio-Tek Instruments Inc.) at 570 nm. Percentage of surviving cells was estimated as: (absorbance at time *t*/initial absorbance) × 100%. Carbidopa showed cytotoxicity significantly greater than L-dopa. It was lethal at 250 μM concentration after 48 h incubation (Fig. 5). However, this effect was not specific for the melanoma cells—similar toxicity was also observed for the control NIH3T3 and HECa10 cell lines (data not shown).

We have also tested the biological activity of the oxidation products of carbidopa by tyrosinase: 3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid and 6,7-dihydroxy-3-methylcinnoline. As expected for catechols, both compounds showed substantial cytotoxicity, although neither was as effective as carbidopa. The dose-dependence profile for 3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid was similar after 24 and 48 h. 6,7-Dihydroxy-3-methylcinnoline was not effective after 24 h but was more potent after 48 h (Fig. 6). Again, however, the effect was not specific to B16(F10) melanoma cells.

Greater cytotoxicity of carbidopa than any of the 3 other tested compounds (L-dopa, 3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid, and 6,7-dihydroxy-3-methylcinnoline) demonstrates that the hydrazine group plays an important role in this process and the effect observed previously in human pulmonary carcinoid and small cell lung carcinoma cells[14,15] is not caused by the catechol moiety alone.

The strong side effects observed during the clinical trials of the combination of carbidopa and L-dopa[17] can most likely be attributed to dopamine formed by decarboxylation of L-dopa. Since the metabolism of carbidopa does not lead to any physiologically significant compounds, it may be worth trying to reevaluate its effect on human melanoma. Modifications of its structure may also lead to compounds with improved efficiency and selectivity.

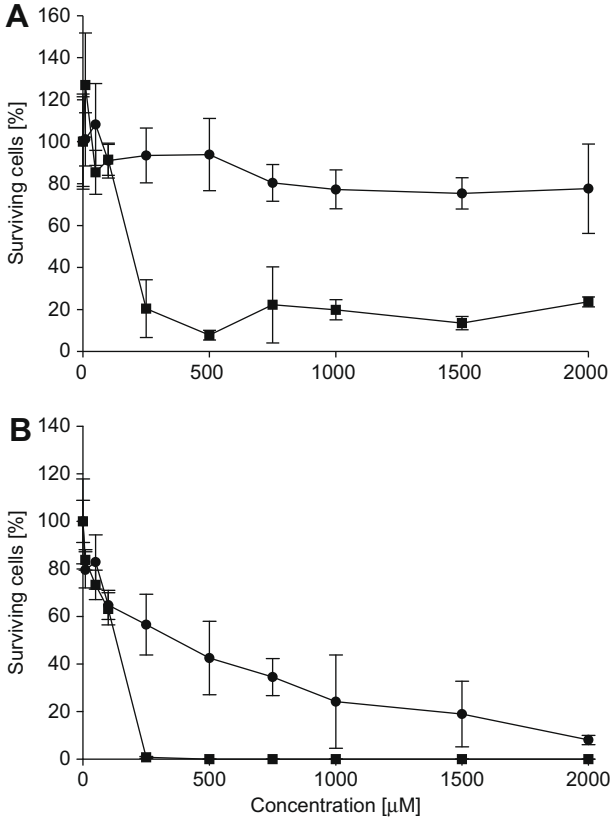


Figure 5. Toxicity of carbidopa (-■-) and L-dopa (-●-) against B16(F10) cells in vitro: A—after 24 h, B—after 48 h.

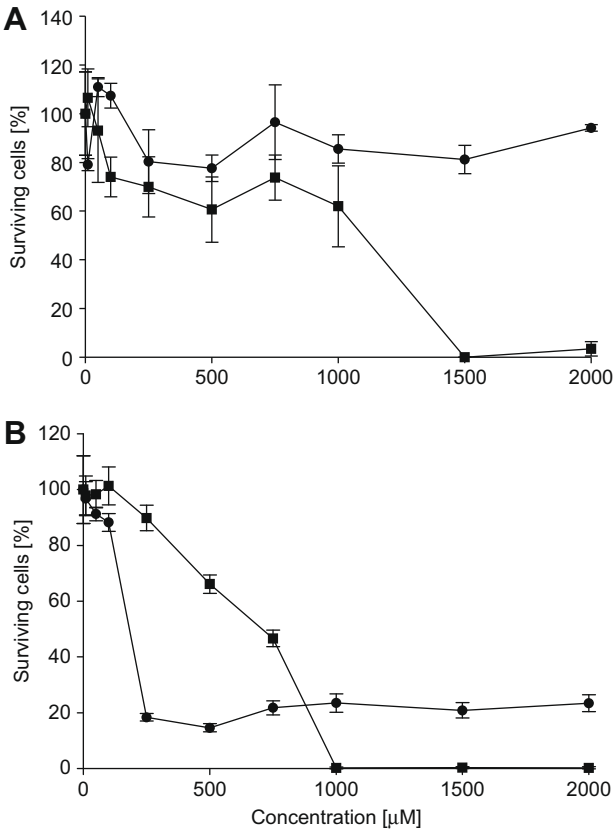


Figure 6. Toxicity of 3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid (-■-) and 6,7-dihydroxy-3-methylcinnoline (-●-) against B16(F10) cells in vitro: A—after 24 h, B—after 48 h.

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