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Cytotoxicity of dopamine-derived tetrahydroisoquinolines on melanoma cells

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Abstract

Tetrahydroisoquinolines (TIQs) are endogenous alkaloid compounds detected in urine, central nervous system and some peripheral tissues in *Mammalia*. No data are at present available on TIQ levels in skin, although *in vitro* biochemical evidences indicate that they may undergo auto-oxidation with production of reactive oxygen species or may be enzymatically converted into melanin pigments. The effect of two catechol-bearing TIQs, salsolinol (SAL) and tetrahydropapaveroline (THP), on the viability of melanotic or amelanotic melanoma cell lines was investigated. Both SAL and THP were well tolerated up to roughly 30 μM and became overtly toxic at higher concentrations, with SAL being better tolerated than THP. Intracellular activity of some antioxidant enzymes, tyrosinase and α-ketoglutarate dehydrogenase was also evaluated to assess the cell response to oxidative and metabolic challenge of TIQs treatment. Catalase and superoxide dismutase pre-treatment only partially prevented TIQs toxicity while a complete protection was obtained with *N*-acetylcysteine and GSH. TIQs were able to provoke upregulation of the scavenging enzymes catalase and DT-diaphorase and to determine a decrease of the α-ketoglutarate dehydrogenase activity. SAL and THP enhanced tyrosinase activity and melanin production, suggesting that they were indeed tyrosinase substrates leading to melanin formation. The results support the evidence that TIQs were toxic toward melanoma cells, leading to their death by necrosis. TIQs toxicity was likely due to increased oxidative stress by generation of reactive oxygen species and oxidative metabolites. Our study represents an intent to furnish an additional contribution for the comprehension of catechol cytotoxicity.

Keywords: Cytotoxicity; Tetrahydroisoquinolines; ROS; Quinones; Melanoma cells

1. Introduction

TIQs are alkaloid compounds deriving from the non-enzymatic Pictet–Spengler condensation of catecholamines with aldehydes [1]. The reaction between dopamine and acetaldehyde entails the generation of SAL, whereas the interaction between dopamine and its derived aldehyde, i.e. 3,4-dihydroxy-phenylacetaldehyde, leads to THP (Scheme 1). In mammals, TIQs are endogenous compounds detected in urine, central nervous system and other peripheral tissues [2,3] and several lines of evidence suggest that these alkaloids are actually involved in biogenic amines regulation by the inhibition of the enzymes engaged in monoamine biosynthesis and degradation [4–9].

No data are available on their levels in skin and other epithelia, although *in vitro* biochemical evidence indicates that TIQs bearing a catecholic moiety are good substrates for tyrosinase, peroxidase, lipoxygenase, xanthine oxidase and ceruloplasmin giving rise to the formation of

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Abbreviations: TIQs, tetrahydroisoquinolines; SAL, salsolinol; THP, tetrahydropapaveroline; ROS, reactive oxygen species; SOD, superoxide dismutase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide: thiazolyl blue; DT-DIA, DT-diaphorase; DCIP, 2,6-dichlorophenol-indophenol; CAT, catalase; GSH-PX, glutathione peroxidase; α-KGDH, α-ketoglutarate dehydrogenase; MBTH, 3-methyl-benzothiazol-2-on-hydrazon hydrochloride; RT, reverse transcriptase; NTP, nucleotide triphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BHT, *tert*-butylated hydroxytoluene; NAC, *N*-acetylcysteine; GSH, glutathione reduced form.

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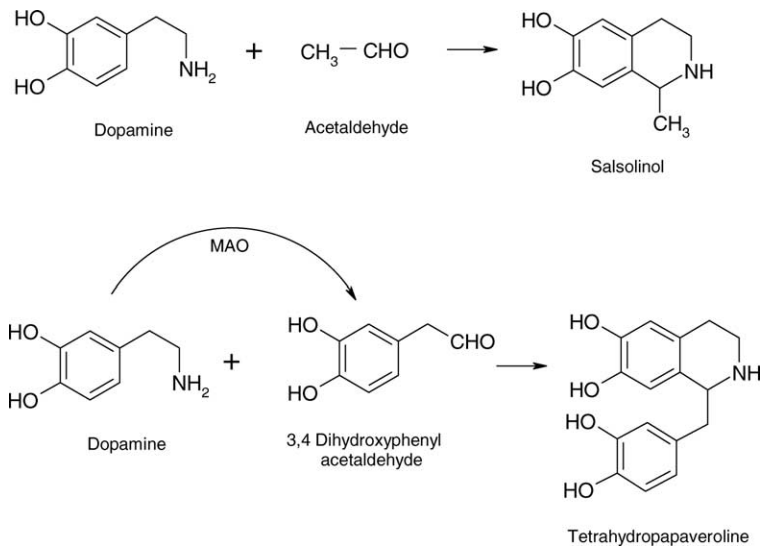

Scheme 1. SAL and THP formation by Pictet–Spengler condensation of dopamine with aldehydes.

TIQ-quinones, TIQ-chromes and ultimately leading to melanin pigments (*TIQ-melanins*) [10–12]. The alkaloids are also converted by auto-oxidation [10] into TIQ-melanins whose scavenging properties have been recently studied and compared with those exerted by dopamelanin and opiomelanins [12,13].

Dopa and related compounds are known as good inhibitors of cyclooxygenase and lipoxygenase, thus, constituting modulator effectors of inflammatory response in many cells [14]. However, the chief challenge represented by melanin precursors is their cytotoxicity, which can be considered a threat for catechol-containing cells and at the same time a useful tool in the strategy of melanoma therapy [15]. The deleterious effect *in vitro* of melanin precursors, i.e. Dopa [16,17], 5-*S*-cysteinyldopa [18] and 5,6-dihydroxyindole [19], has been long known.

The first step of melanogenesis involves the formation of a quinone which through the transient production of leucodopachrome is converted into dopachrome. This intermediate can form either 5,6-dihydroxyindole or 5,6-dihydroxyindol-2-carboxylic acid that are polymerised into melanin pigments.

During their auto- or enzymatic oxidation, catechols liberate reactive oxygen species (ROS) production, together with the nucleophylic addition of crucial thiol protein residues by *o*-quinones, have been suggested as the toxic mechanisms chiefly involved in the harmful activity of catechol compounds [20–23]. The quinone redox-cycling, i.e. the one-electron reduction of quinone to semiquinone and the subsequent auto-oxidation of semiquinone to the quinone, is able to release large quantities of superoxide anion [24] which in turn can—spontaneously or by superoxide dismutase (SOD) action—be transformed into hydrogen peroxide. The last compound through the Fenton system is readily decomposed giving rise to $OH^{\bullet}$, which represents a radical with devastating action on practically every cell components and organelles. By several authors, however, the hydrogen peroxide produced by the overall described processes has to be considered the cardinal agent responsible for cellular killing by catechol overproduction or overesposition [17,25,26].

In the present paper, we present the effects of catechol-bearing TIQs on several lines of melanoma cells, with the intent to give a further contribution for the comprehension of catechol cytotoxicity, also by comparing the data with those simultaneously obtained by cell exposure to Dopa.

2. Materials and methods

2.1. Chemicals

SAL, THP, MTT, DCIP, dicumarol, MBTH, CAT, SOD, α-tocopherol, BHT, NAC, GSH and all culture media were purchased from Sigma Chemical Co. Superscript one-step RT-PCR Platinum TAQ reaction mixture was from GIBCO-BRL Ltd. NTPs were furnished by Roche Diagnostics SpA. Soluene 350 was obtained from Packard Instruments. All other reagents were analytical grade products.

2.2. Cell lines and culture conditions

Four established cell lines were used in the present study: FRM, MNT and M14 are human melanoma cells and B16 are of murine origin. All the lines were kindly provided by Dr. G. Zupi (Laboratory of Chemotherapy, Regina Elena Institute for Cancer Research, Rome). FRM appear as large, colourless fuse-shaped cells, while MNT is a cell line intensely dark with a star-like morphology; both lines have been recently established from melanoma patients, while M14 is a long established melanoma line. B16, M14 and FRM were grown in RPMI 1640 medium

with 10% (v/v) fetal calf serum and sub-cultured twice a week at 1:10, 1:5 and 1:3 ratio, respectively. MNT were maintained in DMEM/ F12 Nutrient Mix 1:1 plus 10% FCS and sub-cultured 1:2 twice a week.

2.3. Cell viability estimation

Cell viability was measured as already described [27]. Briefly, cells were seeded in 96-well microplates at a density which allowed an exponential growth rate for the following 5 day incubation (i.e. 5×10^3/well for B16; 1.0×10^4 for M14 and 1.6×10^4 for FRM and MNT). After overnight incubation the medium was discarded and replaced with fresh medium containing SAL, THP or Dopa at concentration ranging from 10 to 200 μM. At intervals of 24 hr the wells were stained with Crystal Violet, the dye was eluted by means of 33% acetic acid and the A_{540} measured in a microplate reader (Labsystem Multiscan MS). The measurement of metabolic activity was performed by means of MTT assay [28]: the cells were stained for 2 hr with MTT (1.25 mg/mL), the dye was removed by isopropanol and the A_{540}/A_{750} ratio measured by a microplate reader. Each condition was assayed in eight parallel independent replicas.

2.4. Apoptosis markers

Genomic DNAs from cells grown on 35-mm plastic dishes and challenged with different TIQs were harvested and assayed according to Apoptotic DNA-Ladder kit (Roche Diagnostic SpA). Cytoplasmic nucleosomes on adherent cells were determined in a Sandwich EIA assay, according to the Cell Death Detection Elisa kit (Roche Diagnostic SpA).

2.5. Enzyme assays

For the determination of enzymatic activities, cells were harvested by trypsine treatment, washed three times with a cold isotonic buffer and lysed either by sonication (10 s twice) or by resuspending and vortexing in the appropriate lysis buffer. The protein concentration in the lysate was determined using the Bio-Rad Protein Assay.

2.5.1. DT-diaphorase (DT-DIA)

Cells were lysed by sonication and enzymatic activity was assayed spectrophotometrically as described by Ernster [29]. The reaction mixture (1 mL) contained 50 mM phosphate buffer, pH 7.5, 0.3 mM NADPH, 0.04 mM DCIP as electron acceptor and 0.07% BSA as activator. Reaction was started by adding cell lysate (10–50 μg protein), after which the reduction of DCIP—with or without 10^{-5} M dicumarol in the reaction mixture—was recorded at 600 nm in a Kontron Uvikon 930 Spectrophotometer. The dicumarol-sensitive part of the activity was the selective measure of DT-DIA activity [29].

2.5.2. Catalase (CAT)

Cells were lysed by sonication and the activity assayed by following the rate of H_2O_2 consumption at 240 nM in a reaction mixture (1 mL) containing 10 mM H_2O_2, 50 mM phosphate buffer, pH 7.0, and 30 μg protein of cell lysate.

2.5.3. Glutathione peroxidase (GSH-PX)

Determination was performed on cell extracts lysed by sonication, using the Glutathione Peroxidase Cellular Activity Assay kit from Sigma.

2.5.4. α-Ketoglutarate dehydrogenase (α-KGDH)

The lysis buffer was that suggested by Park *et al.* [30]. Enzymatic activity was measured according to Pekovic *et al.* [31], with minor modification. The reaction mixture (1 mL) contained 50 mM Tris–HCl buffer, pH 8.0, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.16 mM coenzyme A, 1 mM NAD, 2.5 mM α-ketoglutarate, 0.5 mg/mL Triton X-100, 0.08 mM rotenone and 30–100 μg protein of cell lysate. NAD reduction was monitored recording fluorescence (460 nm λ_{em}, 358 nm λ_{ex}) in a Jasco FP 770 Spectrofluorometer.

2.5.5. Tyrosinase

Cells were lysed in the buffer described by Jara *et al.* [32]. Enzyme activity was assayed according to the method described by Winder and Harris [33], using MBTH to trap dopaquinone formed by L-Dopa oxidation. The reaction mixture (1 mL) contained 50 mM Na-phosphate buffer, pH 7.1, 2% (v/v) *N*,*N*′-dimethylformamide, 1 mM L-Dopa, 6 mM MBTH and 30 μg protein of cell lysate. The initial rate of MBTH adduct formation was measured by monitoring the absorbance increase at 505 nm.

2.6. Melanin determination

The amount of melanin was assayed according to Ancans and Thody [34] by dissolving a washed cell pellet directly in 1 mL Soluene and incubating the samples for 2 hr at 60°. Melanin absorbance was measured spectrophotometrically at 475 nM.

2.7. RT-PCR

Total DNA and RNA were simultaneously extracted from cell cultures by the Tri-Reagent commercial kit (Molecular Research Centre, Inc.) according to the supplier instruction. The quality of RNA was tested by the A_{260}/A_{280} ratio and by 1.5% agarose gel electrophoresis.

One microgram of total RNA was reverse transcribed and amplified in a 50 μL volume of Superscript One-Step RT-PCR Platinum TAQ reaction mixture, completed with 500 nM upstream and downstream primers and 1.5 mM Mg^{2+}. The reverse transcription was carried out at 45° for 30 min. Samples were then heated to 95° for 150 s to inactivate RT and to activate Platinum TAQ Polymerase. Amplification consisted in 35 cycles with annealing at 52°

for 30 s, extension at 73° for 60 min and denaturation at 95° for 45 s and a final cycle with a 5 min long extension. When nested PCR was performed, 2 μL of the outer reaction were transferred in a 50 μL final volume containing 500 nM each nested primer, 1.5 mM $MgCl_2$, 200 mM each NTP and 2 U/100 μL Platinum TAQ. For human tyrosinase outer and nested primers were HuTYR1 and HuTYR2 [35]. For mouse tyrosinase primers were those indicated by Tsukamoto *et al*. [36] with annealing temperature of 56°. Human and mouse GAPDH was detected by simple RT-PCR with primers and conditions as published [36,37].

3. Results

3.1. Effect on cell viability

The dose–response curve of cell viability after long-term treatment with SAL or THP is shown in Fig. 1A. The results obtained by Dopa exposure are also reported for comparison. Though some differences among the various strains could be registered, generally both alkaloids were well tolerated up to roughly 30 μM becoming more toxic at higher concentrations (100 μM). At 200 μM the

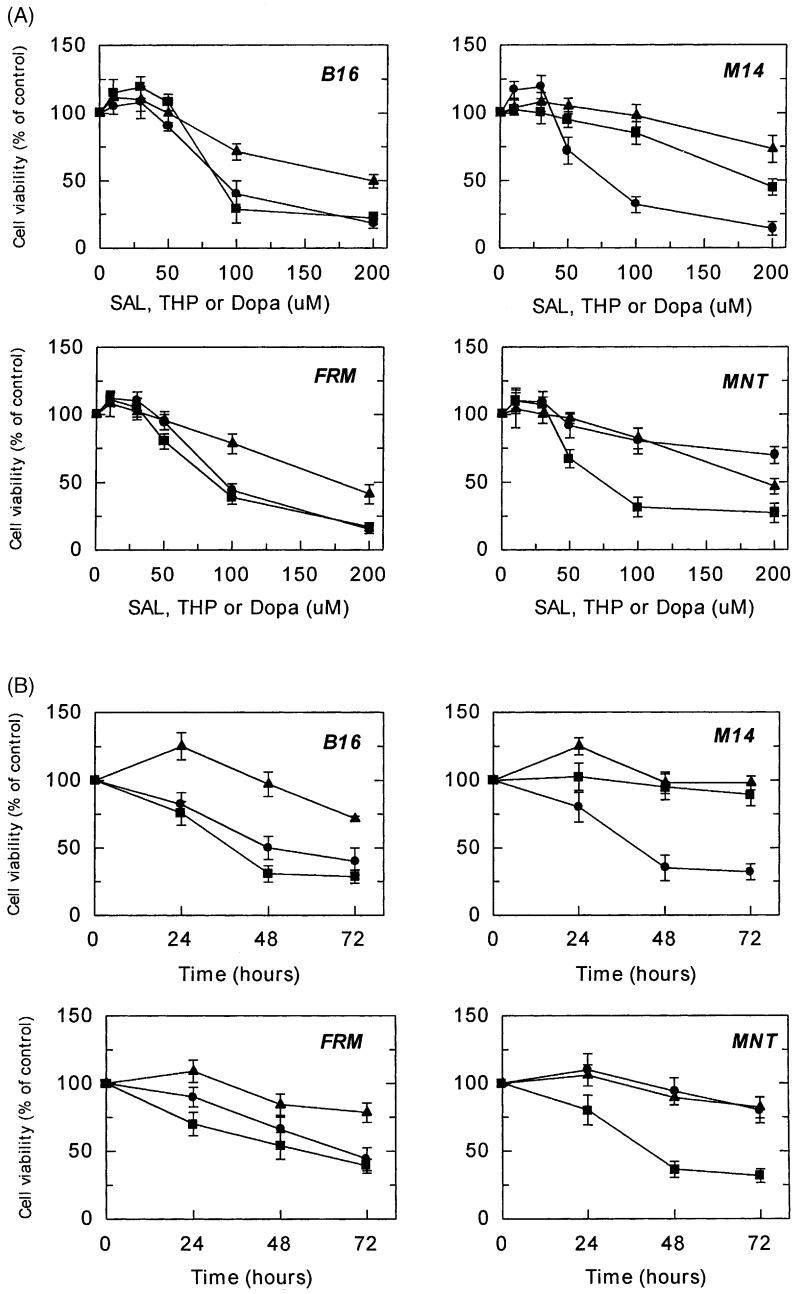

Fig. 1. Effect of SAL (●), THP (■) or Dopa (▲) on melanoma cells viability. (A) Concentration-dependent effect: B16, M14, FRM and MNT cells were treated for 72 hr with SAL, THP or Dopa at the indicated concentrations. (B) Time-dependent effect: B16, M14, FRM and MNT cells were treated for the indicated times with 100 μM SAL, THP or Dopa. The reported values represent cell viability—assessed by the MTT method—expressed as percent of respective controls (i.e. untreated cells of each cell line) and are the mean ± SD of eight independent experiments.

Table 1
Effect of SAL, THP and Dopa on melanoma cells viability

	B16	M14	FRM	MNT
SAL	91.6 ± 3.0	71.0 ± 2.6	93.6 ± 2.2	>200
THP	84.6 ± 1.9	>200	84.9 ± 1.7	73.4 ± 2.7
Dopa	200.0 ± 10	>200	178.3 ± 8.4	200 ± 9.0

Cells were treated for 72 hr with SAL, THP or Dopa at variable concentrations (10–200 μM). The reported data are the IC_{50} values—expressed as micromoles—calculated from the toxicity curves. Data represent the mean ± SD of eight independent experiments.

compounds—with the exception of SAL toward MNT—were significantly toxic.

Dopa was undoubtedly endowed with a minor cytotoxicity than the two alkaloids: generally THP appeared to be more powerful that the other two substances and the maximum difference in the cytotoxic action was reached at 100 μM, whereas at 200 μM saturation profiles were evidenced.

At 100 μM concentration, the cell viability percentages with respect to the controls were in FRM approximately 45, 40 and 80 for SAL, THP and Dopa, respectively, whereas in MNT 85, 30 and 80 values were obtained. This concentration (100 μM) was preferentially chosen in the subsequent experiments to assess the other TIQs effects on melanoma cells. IC_{50} values—obtained from the toxicity curves by non-linear regression analysis—are summarised in Table 1. It is evident that more than a double amount of Dopa was to be administered to obtain the same effect of the alkaloids.

The time course of cell viability upon treatment with 100 μM SAL, THP or Dopa is shown in Fig. 1B. The toxicity of the three compounds was time-dependent and a significant reduction of viable cells was noted after 24 hr (70–80% of untreated controls), while the maximum effect was generally reached after 72 hr.

To evaluate the mechanisms of cell loss, cultures were assayed for the formation of DNA-ladder and cytosolic nucleosomes under TIQ treatment. No elevation of both indicators could be ever shown suggesting that no apoptosis mechanisms are involved in the observed cytotoxicity (data not shown).

3.2. *Effect of enzymatic or non-enzymatic antioxidants on TIQs toxicity*

In order to establish whether the observed toxicity could be due to extracellular hydrogen peroxide or oxygen-derived radicals, TIQs were administered after supplementation of culture media either with 100 mU/mL CAT or 100 mU/mL SOD. Fig. 2A shows that pre-treatment of B16

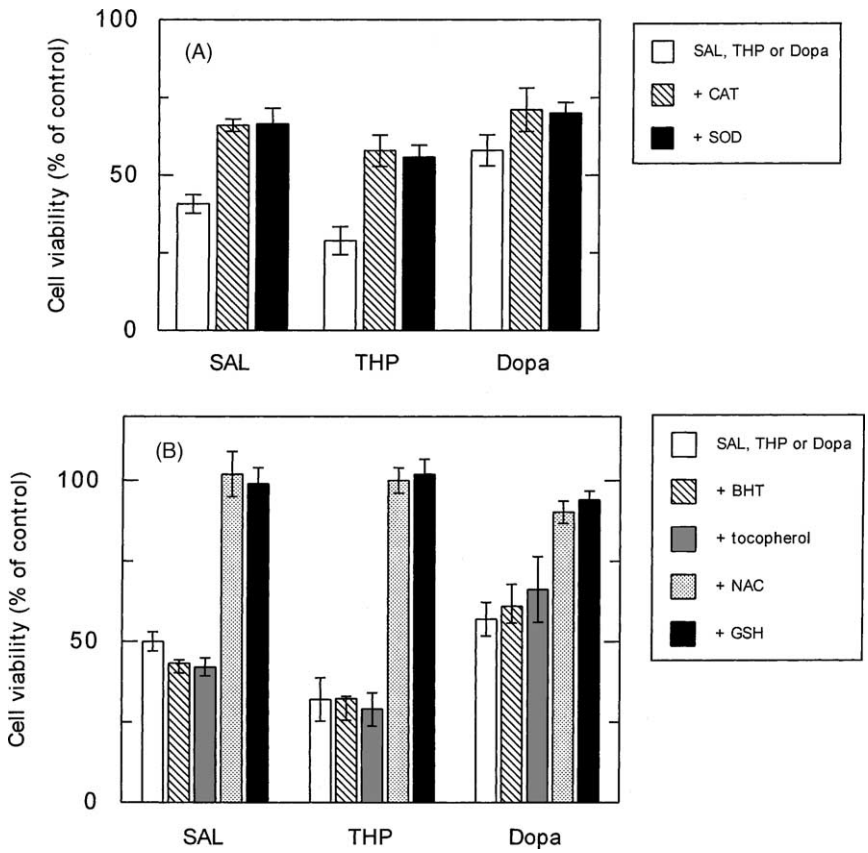

Fig. 2. Effect of enzymatic or non-enzymatic antioxidants on the viability of melanoma cells treated with SAL, THP or Dopa. B16 cells were treated for 48 hr with 100 μM SAL or THP or 200 μM Dopa in the presence of (A) 100 mU/mL CAT or SOD or (B) 50 μM BHT, 10 μM α-tocopherol, 1 mM NAC or 1 mM GSH. The reported values represent cell viability—assessed by the MTT method—expressed as percent of respective controls (i.e. untreated cells of each cell line) and are the mean ± SD of eight independent experiments.

cells with the indicated enzymes prevented only partially the toxicity caused by 48 hr treatment with 100 μM SAL or THP. In fact, when the viability percentage was 41% (SAL exposure) and 29% (THP exposure), antioxidant enzymes supplementation caused a retrieval of 66 and 58% for SAL and THP, respectively. CAT and SOD pre-treatment also induced a partial protection against toxic concentrations of Dopa. For the other cellular lines analogous results were obtained (not shown).

The ability of antioxidant compounds to prevent cell death by TIQs was also evaluated. The effects of BHT, α-tocopherol, NAC and GSH on the viability of B16 cells are shown in Fig. 2B. Cell pre-treatment with BHT or α-tocopherol caused no reversal of the toxic effects; conversely a complete restoration of viability for both SAL- and THP-treated cells was achieved by NAC and GSH.

NAC and GSH also induced an almost complete protection of Dopa-treated cells (about 80% in FRM and 90% in B16) though the catechol was administered at 200 μM concentration in order to provoke an appreciable toxic effect. For the other cell lines similar results were obtained (not shown).

3.3. Intracellular activity of antioxidant enzymes

In order to give an evidence of cell response to oxidative stress, the intracellular activity of some antioxidant enzymes such as DT-DIA, CAT, GSH-PX and tyrosinase was evaluated. DT-DIA (NADP(H): quinone oxidoreductase EC 1.6.99.2) is an inducible flavoenzyme arising upon xenobiotics administration or quinone production [38] which has been found in melanocytes by Smit *et al.* [39]. For its ability in reducing quinones and acting as a SOD-like enzyme for the superoxide scavenging capacity, DT-DIA is regarded as an antioxidant enzyme [38]. Table 2 shows that exposure of the two amelanotic cell lines M14 and FRM to SAL or THP induced an increase in CAT and DT-DIA activity (particularly evident in M14), whereas GSH-PX activity remained unaltered. In the melanotic MNT and B16 strains, DT-DIA and GSH-PX remained also unchanged by TIQs treatment, whereas a decrease of CAT activity was actually evidenced. In most of the experiments, the effects elicited by THP exposure were more pronounced than those exerted by SAL.

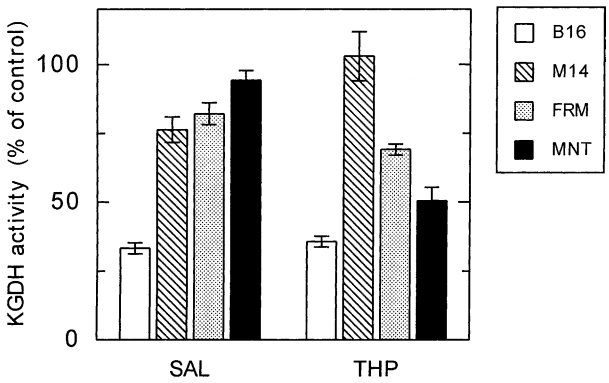

Fig. 3. Effect of SAL or THP on the α-KGDH activity of melanoma cells. Cells were treated for 48 hr with 100 μM SAL or THP. The α-KGDH activity was assayed in the cell lysates by monitoring the increase of fluorescence at 358 nM λ_{ex} and 460 nM λ_{em}. The reported values represent enzymatic activity expressed as percent of respective controls (i.e. untreated cells of each cell line) and are the mean ± SD of five independent experiments.

3.4. α-KGDH activity

In order to assess TIQs effect on mitochondrial metabolism, α-KGDH activity was also determined (Fig. 3). With the exception of M14 THP treated and MNT SAL treated, whose α-KGDH activity was unaltered respect to the control, a general decrease of enzymatic activity was evidenced. In particular, a very strong diminution (about 70% of control) was found in B16 cells, whose viability was highly impaired by TIQs treatment. This correspondence might suggest a direct correlation between α-KGDH residual activity and cell surviving.

3.5. Tyrosinase activity and mRNA expression

The effects of TIQs exposure on tyrosinase activity are shown in Table 3. While no activity was detectable in the two amelanotic lines (M14 and FRM), an increase was observed in B16 and MNT melanotic lines, both by SAL and THP treatment; only in MNT SAL-treated cells tyrosinase levels were equal to the control. In accordance, melanin content showed an increment in SAL- and THP-treated B16 and in THP-treated MNT cells.

To investigate the reason for the lack of tyrosinase activity in M14 and FRM cells, an RT-PCR assay for

Table 2
Effect of SAL and THP on the activity of some antioxidant enzymes

	B16		M14		FRM		MNT	
	SAL	THP	SAL	THP	SAL	THP	SAL	THP
CAT	78.3 ± 4.1	73.5 ± 3.5	99.2 ± 2.5	121.4 ± 12	120 ± 10	126.3 ± 7	67.3 ± 7.2	84.2 ± 8.9
DT-DIA	–[a]	–[a]	135 ± 6.3	156.0 ± 10	117 ± 4.5	115 ± 8.4	100 ± 3.6	112 ± 8.1
GSH-PX	–[a]	–[a]	60.2 ± 5.8	81.2 ± 7.1	113 ± 7.9	95 ± 6.6	101 ± 4.8	93.1 ± 5.3

Cells were treated with 100 μM SAL or THP for 48 hr. The reported values represent the enzymatic activities expressed as percent of respective controls (i.e. untreated cells of each cell line) and are the mean ± SD of five independent experiments.

[a] The murine B16 line lacks DT-DIA and GSH-PX activities.

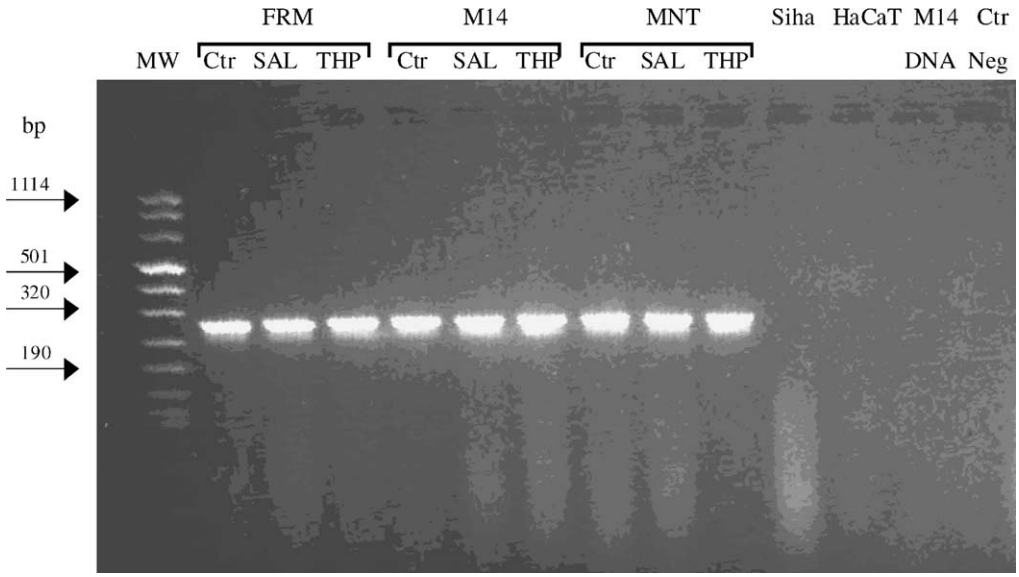

Fig. 4. Tyrosinase mRNA expression in melanotic and amelanotic cell lines. One microgram of total RNA was reverse transcribed and amplified with HuTyr1/HuTyr2 as described in the text. A marked band was detected in any cell line both under control and TIQs treatments. As expected Siha and HaCaT epithelial cells, devoid of tyrosinase, do not show any signal. No signal was also detected in genomic DNA from M14 cells. Ctr Neg: reaction negative control. MW: molecular weight marker. Arrows on the left side indicate molecular weight in base pairs of standard bands.

Table 3
Effect of SAL and THP on tyrosinase activity and melanin content

	B16		MNT	
	SAL	THP	SAL	THP
Tyrosinase activity	140 ± 13.1	128 ± 8.5	103 ± 1.5	138.5 ± 15.5
Melanin content	160 ± 18.2	140 ± 15.0	123 ± 7.5	325 ± 14.0

Cells were treated with 100 μM SAL or THP for 48 hr. Tyrosinase activity was assayed in the cell lysates by determining the rate of MBTH adduct formation, monitored at 505 nM. For melanin determination, washed cell pellets were directly dissolved in Soluene; after incubation for 2 hr at 60°, melanin absorbance was measured at 475 nM. The reported values are expressed as percent of respective controls (i.e. untreated cells of each cell line) and represent the mean ± SD of eight independent experiments.

tyrosinase mRNA was performed. As it can be seen in Fig. 4, both cell lines expressed remarkable levels of tyrosinase mRNA either under TIQs treatment or in control conditions. Moreover, their mRNA levels were comparable with those of the intensely melanotic MNT line.

4. Discussion

When TIQs were administered at low concentrations (10–30 μM), a mild but evident proliferative effect was observed. This phenomenon, already reported by literature, can be probably ascribed to ROS that—at low concentrations—act as messengers for triggering mitotic activity [40].

At higher concentration, THP and SAL proved to be highly toxic for melanoma cells in concentration- and time-dependent manner. In any condition, TIQs were more harmful than Dopa itself, as displayed by the IC_{50} values which documented that a double amount of Dopa was necessary to elicit the same cytotoxic effect exerted by TIQs.

TIQs have been found to promote ROS production *in vitro* and *in vivo* [41–43] during auto-oxidation and it has been also reported that SAL—as, on the other hand, Dopa [44,45]—undergoes redox-cycling through the catechol moiety, thereby generating ROS that can mediate cytotoxicity [46]. When our cell strains were pre-treated with CAT and SOD, a partial protection was observed, indicating that hydrogen peroxide and superoxide anion are only partially responsible for the oxidative stress and that other toxic mechanisms have to be invoked to explain alkaloids action.

α-Tocopherol and BHT were not competent in reversing the harmful effects of TIQs, in contrast with the capacity exerted by NAC and GSH which completely counteracted the TIQs deleterious behaviour. This behaviour seems to indicate that the major damage caused by TIQs occurs *via* interference with cellular SH homeostasis. Indeed, NAC and GSH, acting as disulphide reducing agents, restore the proper ratio GSH/GSSG and protect melanoma cells by the adverse effects of toxicants able to cause GSH depletion [47].

Actually thiol compounds can achieve cell rescue and survival by different mechanisms: (i) they have the ability to scavenge radicals eventually formed by TIQs and catechols auto- or enzymatic oxidation, (ii) they can react with both catechol- or TIQ-quinones by forming cysteinyl adducts [48] and (iii) they can directly increase intracellular GSH content [47].

The overall determination of intracellular antioxidant enzymes provided an indication that the TIQ-generated noxious stimuli were able to provoke DT-DIA and CAT

upregulation whereas GSH-PX remained substantially unchanged. These data are in line with previous investigations showing the ability of both Dopa and 4-hydroxyanisole in inducing DT-DIA in astroglial and melanoma cells [39,49].

We also showed that TIQs provoke an increase of tyrosinase activity and a parallel enhancement of melanin content in the cells. The result is not fully unexpected if one considers the ability of TIQs to be oxidised *in vitro* to melanin pigments by tyrosinase and by a series of oxidative enzymes converted into peroxidative agents in the presence of hydrogen peroxide [10,12] and that both tyrosine and Dopa are recognised as positive regulators of melanogenesis by tyrosinase stimulation in melanoma cells [50]. The parallel enhancement of tyrosinase and melanin suggests that intracellular TIQs effects may involve tyrosinase expression with the consequent production of melanin.

A series of investigation has revealed that tyrosinase in pigment cells is not always in a catalytically active state; our experiments demonstrated that tyrosinase mRNA was actually present also in those cells where no tyrosinase activity was detected. This result points to a post-transcriptional inhibition mechanism and confirms that the levels of mRNA or the abundance of the enzyme does not reflect the tyrosinase activity in cell cultures [51,52].

Because it has been established that tyrosinase can preferentially utilise the anion superoxide instead of oxygen for producing melanin [53,54], tyrosinase may be enrolled among the scavenging enzymes. In melanoma cells, melanin formation may be regarded as a more relevant strategy for eliminating ROS than antioxidant enzymes such as CAT, DT-DIA and GSH-PX whose activity actually remains unaltered upon TIQs exposure. This suggestion is in keeping with previous data reported by Valverde *et al*. [55].

The ability of TIQs in decreasing α-KGDH activity indicates that their toxicity is also exerted at the mitochondrial level. As α-KGDH catalyses the oxidation of α-ketoglutarate to succinate, which serves as substrate for complex II, the inhibition of the former enzyme can impair electron transfer *via* complex II. An involvement of complex I can also be envisaged because the conversion of α-ketoglutarate produces NADH which enters the mitochondrial chain *via* complex I. Though a mechanism of down-regulation caused by TIQs or TIQs metabolites cannot be excluded, the decreased activity of the α-KGDH can be also imputed to a direct inhibition of the enzyme, because this event has been also directly ascertained *in vitro* on mitochondrial preparations [56]. A direct toxic effect of the TIQs on the mitochondrial chain with a derangement of energy supply may also be hypothesised: indeed catechol- or TIQ-quinones, as well as ROS, could easily react with the sulfhydryl groups of lipoic acid present as a coenzyme in α-KGDH complex. On the other hand, a similar action has been reported for Dopa and dopamine which exert a toxic activity by inhibiting mitochondrial respiration and some enzymatic complexes at the mitochondrial level in neuroblastoma and pheochromocytoma cell lines [57,58]. Our suggestions are also in accordance with those reported by Storch *et al*. [59], who proposed the impairment of cellular energy metabolism as a cause of SAL toxicity. Indeed, for their ability to inhibit mitochondrial enzymes and because they have been retrieved in human Parkinsonian brain [3,60] and in rat brain during ethanol intoxication [61], TIQs are suspected of being associated with the etiology of Parkinson's disease and in the neuropathology of chronic alcoholism [60–64].

The DNA-ladder and the colorimetric assay indicated that toxic doses of TIQs provoke cell death by necrosis rather than apoptosis. This event is in keeping with previous experiments by Maruyama *et al*. [65] who suggested necrosis as the type of cell death after treatment of neuroblastoma cells with TIQs. Conversely, Dopa and dopamine have been shown to induce apoptosis in a variety of mammalian cells [66,67]; however it seems to be no clear-cut border line between the two events, mainly depending by both stress intensity and ATP levels [68,69].

In conclusion, the interaction of TIQs with melanoma cells can be depicted as follows:

1. In a first instance, hydrogen peroxide and ROS produced prevalently by auto-oxidation at submicromolar TIQ levels can act as messengers by promoting growth response and stimulating antioxidant defences by upregulation of scavenging enzymes such as CAT, DT-DIA and tyrosinase;
2. By this response, H_2O_2, ROS and quinones can be partially removed and tyrosinase provides for both superoxide elimination and melanin synthesis, which offers a further shield to noxious stimuli;
3. When TIQs are present at high concentration, cells undergo an oxidative stress, due to continuous production of radical species that saturate cell reducing capabilities;
4. This oxidative stress is not removed by the detoxifying enzymes and impairs the mitochondrial functions through α-KGDH, ultimately leading to cell damage and death.

Based on these findings, it seems likely that TIQs undergo redox-cycling with concomitant production of ROS and reactive intermediates, including *o*-quinones which can interfere with thiol homeostasis by addition reactions, thereby inducing cell death by necrosis.

A considerable attention has been directed in the past years on the possibility of exploiting melanogenic precursors as antimelanoma agents; several catechols and especially phenolic prodrugs have been developed and some of these have reached a preclinical stage [15]. A better comprehension of toxicological mechanisms of catechols or TIQs may be a useful insight for novel advances in model systems endeavour for increasing the antimelanoma defensive armamentary.

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References

[1] Dietrich R, Erwin V. Biogenic amine–aldehyde condensation products: tetrahydropapaverolines and tryptolines. Ann Rev Pharmacol Toxicol 1980;20:55–80.
[2] Musshoff F, Daldrup T, Bonte W, Leitner A, Lesch OM. Salsolinol and norsalsolinol in human urine samples. Pharmacol Biochem Behav 1997;58:545–50.
[3] Niwa T, Takeda N, Kaneda N, Hashizume Y, Nagatsu T. Presence of tetrahydroisoquinoline and 2-methyl tetrahydroisoquinoline in Parkinsonian and human brains. Biochem Biophys Res Commun 1987; 144:1084–9.
[4] Collins AC, Cashaw JL, Davis VE. Dopamine derived tetrahydroisoquinolines alkaloids inhibitors of neuroamine metabolism. Biochem Pharmacol 1973;22:2337–48.
[5] Minami M, Takahashi T, Maruyama W, Takahashi A, Dostert P, Nagatsu T, Naoi M. Inhibition of tyrosine hydroxylase by *R* and *S* enantiomers of salsolinol, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline. J Neurochem 1992;58:2097–101.
[6] Minami M, Maruyama W, Dostert P, Nagata T, Naoi M. Inhibition of type A and B monoamine oxidase by 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolines and their *N*-methylated derivatives. J Neural Transm Gen Sect 1993;92:125–35.
[7] Giovine A, Renis M, Bertolino A. In vivo and in vitro studies on the effect of tetrahydropapaveroline and salsolinol on COMT and MAO activity in rat brain. Pharmacology 1976;14:86–94.
[8] Cohen G, Katz S. 6,7-Dihydroxytetrahydroisoquinoline: evidence for in vivo inhibition of intraneuronal monoamine oxidase. J Neurochem 1975;25:719–22.
[9] Thull U, Kneubuhler S, Gaillard P, Carrupt PA, Testa B, Altomare C, Carotti A, Jenner P, McNaught KS. Inhibition of monoamine oxidase by isoquinoline derivatives. Qualitative and 3D-quantitative structure–activity relationships. Biochem Pharmacol 1995;50: 869–77.
[10] Rosei MA, Mosca L. Production of melanin pigments by chemical and enzymatic oxidation of tetrahydroisoquinolines. Biochem Mol Biol Int 1995;35:1253–9.
[11] Rosei MA, Foppoli C, Wang XT, Coccia R, Mateescu MA. Production of melanins by ceruloplasmin. Pigment Cell Res 1998;11:98–102.
[12] Mosca L, Blarzino C, Coccia R, Foppoli C, Rosei MA. Melanins from tetrahydroisoquinolines. Spectroscopic characteristics, scavenging activity and redox transfer properties. Free Radic Biol Med 1998;24:161–7.
[13] Rosei MA. Opiomelanins synthesis and properties. Histol Histopathol 2001;16:931–5.
[14] Napolitano A, Palumbo A, Misuraca G, Prota G. Inhibitory effect of melanin precursors on arachidonic acid peroxidation. Biochim Biophys Acta 1993;1168:175–80.
[15] Riley PA. New approaches to the utilization of the melanogenic, pathway as a targeting strategy in antimelanoma therapy. In: Hori Y, Hearing VJ, Nakayama J, editors. Melanogenesis and malignant melanoma: biochemistry, cell biology, molecular biology, pathophysiology, diagnosis and treatment. Amsterdam: Elsevier, 1996. p. 271–5.
[16] Wick MM, Byers L, Frei E. L-Dopa: selective toxicity for melanoma cells in vitro. Science 1977;197:468–9.
[17] Urabe K, Aroca P, Tsukamoto K, Mascagna D, Palumbo A, Prota G, Hearing V. The inherent cytotoxicity of melanin precursors: a revision. Biochim Biophys Acta 1994;1221:272–8.
[18] Ito S, Inoue S, Fujita K. The mechanism of toxicity of 5-*S*-cysteinyldopa to tumour cells. Hydrogen peroxide as a mediator of cytotoxicity. Biochem Pharmacol 1983;32:2079–81.
[19] Pawelek JM, Lerner AB. 5,6-Dihydroxyindole is a melanin precursor showing a potent cytotoxicity. Nature 1978;276:627–8.
[20] Prota G. Melanins and melanogenesis. San Diego: Harcourt Brace Jovanovich Publishers/Academic Press, Inc., 1992.
[21] Wardman P. Bioreductive activation of quinones: redox properties and thiol reactivity. Free Radic Res Commun 1990;8:219–29.
[22] Graham DG. Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. Mol Pharmacol 1978;14:633–43.
[23] Montine TJ, Picklo MJ, Amarnath V, Whetsell Jr WO, Graham DG. Neurotoxicity of endogenous cysteinylcatechols. Exp Neurol 1997;148:26–33.
[24] Kappus H, Sies H. Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. Experientia 1981;37: 1233–41.
[25] Karg E, Rosengren E, Rorsman H. Hydrogen peroxide as a mediator of dopac-induced effects on melanoma cells. J Invest Dermatol 1991;96: 224–7.
[26] Passi S, Picardo M, Nazzaro-Porro M. Comparative cytotoxicity of phenols in vitro. Biochem J 1987;245:537–42.
[27] De Marco F, Di Lonardo A, Venuti A, Marcante ML. Interferon inhibition of neoplastic phenotype in cell lines harbouring human papillomavirus sequences. J Biol Regul Homeost Agents 1991;5:65–70.
[28] Gerlier D, Thomasset N. Use of MTT colorimetric assay to measure cell activation. J Immunol Methods 1986;94:5763–9.
[29] Ernster L. DT-Diaphorase. In: Eastbrook RW, Pullman ME, editors. Methods in enzymology, vol. 10. New York: Academic Press, 1967. p. 309–17.
[30] Park LC, Zhang H, Sheu KF, Calingasan NY, Kristal BS, Lindsay JG, Gibson GE. Metabolic impairment induces oxidative stress, compromises inflammatory responses, and inactivates a key mitochondrial enzyme in microglia. J Neurochem 1999;72:1948–58.
[31] Pekovich SR, Martin PR, Singleton CK. Thiamine deficiency decreases steady-state transketolase and piruvate dehydrogenase but not α-ketoglutarate dehydrogenase mRNA levels in three human cell types. J Nutr 1998;128:683–7.
[32] Jara JR, Solano F, Lozano AL. Assays for mammalian tyrosinase: a comparative study. Pigment Cell Res 1988;1:332–9.
[33] Winder AJ, Harris H. New assays for the tyrosine hydroxylase and dopa oxidase activities of tyrosinase. Eur J Biochem 1991;198: 317–26.
[34] Ancans J, Thody AJ. Activation of melanogenesis by vacuolar type H^+-ATPase inhibitors in amelanotic, tyrosinase positive human and mouse melanoma cells. FEBS Lett 2000;478:57–60.
[35] Calogero A, Timmer-Bosscha H, Schrafford Koops H, Tiebosch AT, Mulder NH, Hospers GA. Limitations of the nested reverse transcriptase polymerase chain reaction on tyrosinase for the detection of malignant melanoma micrometastases in lymph nodes. Br J Cancer 2000;83:184–7.
[36] Tsukamoto K, Hirata S, Osada A, Kitamura R, Shimada S. Detection of circulating melanoma cells by RT-PCR amplification of three different melanocyte-specific mRNAs in a mouse model. Pigment Cell Res 2000;13:185–9.
[37] Dondi E, Pattyn E, Lutfalla G, Van Ostade X, Uze G, Pellegrini S, Tavernier J. Down-modulation of type 1 interferon responses by receptor cross-competition for a shared JAK kinase. J Biol Chem 2001;276:47004–12.
[38] Ernster L. DT-Diaphorase: its structure, function, regulation and its role in antioxidant defence and cancer chemotherapy. In: Yagi K, editor. Pathophysiology of lipid peroxides and related free radicals. Tokyo, Japan: Sc Sco Press, 1967. p. 1–24.

[39] Smit NP, Hoogduijn MJ, Riley PA, Pavel S. Study of DT-diaphorase in pigment-producing cells. Cell Mol Biol 1999;45:1041–6.
[40] Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Radic Biol Med 1995;18:775–94.
[41] Maruyama W, Dostert P, Matsubara K, Naoi M. *N*-Methyl salsolinol produces hydroxyl radicals: involvement to neurotoxicity. Free Radic Biol Med 1995;19:67–75.
[42] Maruyama W, Dostert P, Naoi M. Dopamine-derived 1-methyl-6, 7-dihydroxyisoquinolines as hydroxyl radical promoters and scavengers in rat brain: in vivo and in vitro studies. J Neurochem 1995;64: 2635–43.
[43] Nappi AJ, Vass E, Collins MA. Contrasting effects of catecholic and *O*-methylated tetrahydroisoquinolines on hydroxyl radical production. Biochim Biophys Acta 1999;1434:64–73.
[44] Basma AN, Morris EJ, Nicklas WJ, Geller HM. L-Dopa cytotoxicity to PC12 cells in culture is via its autoxidation. J Neurochem 1995;64: 825–32.
[45] Nappi AJ, Vass E. Comparative studies of enhanced iron-mediated production of hydroxyl radical by glutathione, cysteine, ascorbic acid, and selected catechols. Biochim Biophys Acta 1997;1336: 295–302.
[46] Jung Y, Surh Y. Oxidative DNA damage and cytotoxicity induced by copper-stimulated redox cycling of salsolinol, a neurotoxic tetrahydroisoquinoline alkaloid. Free Radic Biol Med 2001;30:1407–17.
[47] Karg E, Tunek A, Brotell H, Rosengren E, Rorsman H. Alteration of glutathione level in human melanoma cells: effect of *N*-acetyl-L-cysteine and its analogues. Pigment Cell Res 1990;3:11–5.
[48] Zhang F, Dryhurst G. Effects of L-cysteine on the oxidation chemistry of dopamine: new reaction pathways of potential relevance to idiopathic Parkinson's disease. J Med Chem 1994;37:1084–98.
[49] van Muiswinkel FL, Riemers FM, Peters GJ, LaFleur MV, Siegel D, Jongenelen CA, Drukarch B. L-Dopa stimulates expression of the antioxidant enzyme NAD(P)H:quinone oxidoreductase (NQO) in cultured astroglial cells. Free Radic Biol Med 2000;29: 442–53.
[50] Slominski A, Moellmann G, Kuklinska E, Bomirski A, Pawelek J. Positive regulation of melanin pigmentation by two key substrates of the melanogenic pathway, L-tyrosine and L-dopa. J Cell Sci 1988; 89:287–96.
[51] Naeyaert JM, Eller M, Gordon PR, Park HY, Gilchrest BA. Pigment content of cultured human melanocytes does not correlate with tyrosinase message level. Br J Dermatol 1991;125:297–303.
[52] Fuller BB, Drake MA, Spaulding DT, Chaudhry F. Downregulation of tyrosinase activity in human melanocyte cell cultures by yohimbine. J Invest Dermatol 2000;114:268–76.
[53] Wood JM, Schallreuter KU. Studies on the reactions between human tyrosinase, superoxide anion, hydrogen peroxide and thiols. Biochim Biophys Acta 1991;1074:378–85.
[54] Valverde P, Manning P, Todd C, McNeil CJ, Thody AJ. Tyrosinase may protect human melanocytes from the cytotoxic effects of the superoxide anion. Exp Dermatol 1996;5:247–53.
[55] Valverde P, Manning P, McNeil CJ, Thody AJ. Activation of tyrosinase reduces the cytotoxic effects of the superoxide anion in B16 mouse melanoma cells. Pigment Cell Res 1996;9:77–84.
[56] McNaught KS, Altomare C, Cellamare S, Carotti A, Thull U, Carrupt PA, Testa B, Jenner P, Marsden CD. Inhibition of alpha-ketoglutarate dehydrogenase by isoquinoline derivatives structurally related to 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP). Neuroreport 1995;6:1105–8.
[57] Pardo B, Mena MA, de Yebenes JG. L-Dopa inhibits complex IV of the electron transport chain in catecholamine-rich human neuroblastoma NB69 cells. J Neurochem 1995;64:576–82.
[58] Offen D, Ziv I, Panet H, Wasserman L, Stein R, Melamed E, Barzilai A. Dopamine-induced apoptosis is inhibited in PC12 cells expressing Bcl-2. Cell Mol Neurobiol 1997;17:289–304.
[59] Storch A, Kaftan A, Burkhardt K, Schwarz J. 1-Methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol) is toxic to dopaminergic neuroblastoma SH-SY5Y cells via impairment of cellular energy metabolism. Brain Res 2000;855:67–75.
[60] Nagatsu T. Isoquinoline neurotoxins in the brain and Parkinson's disease. Neurosci Res 1997;29:99–111.
[61] Collins MA, Bidgeli MG. Biosynthesis of tetrahydroisoquinoline alkaloids in brain and other tissues of ethanol-intoxicated rats. Adv Exp Med Biol 1975;59:79–91.
[62] Dostert P, Strolin Benedetti M, Dordain G. Dopamine-derived alkaloids in alcoholism and in Parkinson's and Huntington's diseases. J Neural Transm 1988;74:61–74.
[63] McNaught KS, Carrupt PA, Altomare C, Cellamare S, Carotti A, Testa B, Jenner P, Marsden CD. Isoquinoline derivatives as endogenous neurotoxins in the aetiology of Parkinson's disease. Biochem Pharmacol 1998;56:921–33.
[64] Sjoquist B, Eriksson A, Winblad B. Salsolinol and catecholamines in human brain and their relation to alcoholism. Prog Clin Biol Res 1982;90:57–67.
[65] Maruyama W, Sango K, Iwasa K, Minami C, Dostert P, Kawai M, Moriyasu M, Naoi M. Dopaminergic neurotoxins 6,7-dihydroxy-1-(3′,4′-dihydroxybenzyl)-isoquinolines, cause different types of cell death in SH-SY5Y cells: apoptosis was induced by oxidized papaverolines and necrosis by reduced tetrahydropapaverolines. Neurosci Lett 2000;291:89–92.
[66] Melamed E, Offen D, Shirvan A, Djaldetti R, Barzilai A, Ziv I. Levodopa toxicity and apoptosis. Ann Neurol 1998;44:S149–54.
[67] Migheli R, Godani C, Sciola L, Delogu MR, Serra PA, Zangani D, De Natale G, Miele E, Desole MS. Enhancing effect of manganese on L-DOPA-induced apoptosis in PC12 cells: role of oxidative stress. J Neurochem 1999;73:1155–63.
[68] Lenaz G. Role of mitochondria in oxidative stress ad ageing. Biochim Biophys Acta 1998;1366:53–67.
[69] Dypbukt JM, Ankarcrona M, Burkitt M, Sjoholm A, Strom K, Orrenius S, Nicotera P. Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells. The role of intracellular polyamines. J Biol Chem 1994;269:30553–60.