

Dopaquinone Addition Products in Cultured Human Melanoma Cells

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The concentrations of dopa, cysteinyl dopas, 5-S-glutathionyl dopa, γ -glutamyl-5-S-cysteinyl dopa and 5-S-cysteinylglycinedopa, were analysed in homogenates of cultured human melanoma cells and in culture media. Cysteinyl dopas were found to be the major catechol in the cells, with a molar concentration more than a hundred times that of dopa. 5-S-Glutathionyl dopa was found in the same amount as dopa, while the quantity of 5-S-cysteinylglycinedopa was one order of magnitude less. γ -Glutamyl-5-S-cysteinyl dopa was not present in detectable amounts. In the medium the concentrations of dopa, 5-S-cysteinylglycinedopa and of 5-S-glutathionyl dopa were about one half of those in the cells, while the concentration of cysteinyl dopas was about 2%. The ratio between 2-S-cysteinyl dopa and 5-S-cysteinyl dopa when incubating dopa and cysteine with tyrosinase was identical with the ratio between the analogically synthesised isomers of glutathionyl dopa. Consequently, from the calculation of these ratios in cells and media one cannot deduce whether cysteinyl dopas arise from the direct addition of cysteine to dopaquinone, or from degradation of glutathionyl dopa. Oxidation of 5-S-glutathionyl dopa gives a red chromophore with maximum absorption at 480 nm which develops into a black pigment. *Key words: Dopa; Melanin; Cysteinyl dopa; Glutathione.* (Received August 22, 1986.)

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During the last few decades the amino acid 5-S-cysteinyl dopa has attracted increasing attention, initially as a marker for pheomelanogenesis, later on as a recorder of malignant melanoma (1). Determinations of its levels in plasma and urine have been used for the diagnosis of metastases from melanomas, even before they become clinically detectable (2).

Subsequently it has been shown that cysteinyl dopas are produced by all melanin producing cells, and the former division between eu- and pheo-melanins has given way to the concept of mixed melanins. It seems that cysteinyl dopa oxidation products are copolymerised with dopaquinone oxidation products in all forms of human melanins (3, 4, 5, 6, 7).

The formation of cysteinyl dopa requires the presence of a thiol in a cellular compartment where dopaquinone is formed, since the lifetime of the latter molecule is very short; dopaquinone is oxidized to indoles when no thiols are present. The thiol can be cysteine, giving cysteinyl dopa directly, or glutathione forming glutathionyl dopa, which compound can then be metabolized to cysteinyl dopa (8). The existence in vivo of both compounds has been documented. Glutathionyl dopa was first demonstrated in malignant melanoma by Agrup et al. (9), who also have shown the metabolism of glutathionyl dopa to cysteinyl dopa by homogenates from melanoma, the enzymes responsible being a gamma-glutamyltranspeptidase and a dipeptidase. The gamma-glutamyltranspeptidase catalysed degradation of glutathionyl dopa should yield cysteinylglycinedopa (8) but this compound has so far not been demonstrated in melanocytes or in human melanoma cells.

Mishima and coworkers have found that the level of gamma-glutamyltranspeptidase is greater in melanotic than in amelanotic melanoma cells, and further that this enzyme is present in premelanosomes, but not in melanosomes (10, 11, 12).

Prota and his colleagues have shown that the levels of glutathionereductase are greater in red than in black guinea pig skin, and from this finding have drawn the conclusion that glutathione plays a role in pigment production (13, 14).

In the present study the occurrence of dopa, of 5-S- and 2-S-cysteinyl-dopa and of 5-S-glutathionyl-dopa has been studied in a well defined human melanoma cell line, IGR 1 (15, 16, 17, 18, 19). In addition 5-S-cysteinylglycinedopa was identified as a metabolite of the melanoma cells (20, 21).

MATERIALS AND METHODS

Cysteinyl-dopas were prepared as previously described (22, 23). Enzyme-catalysed synthesis of cysteinylglycinedopas and glutathionyl-dopas were performed using methods originally developed for the preparation of cysteinyl-dopas (22, 23). Since cysteinylglycine was not available in reduced form it was prepared from cystinyl-diglycine.

Preparation of 5-S-L-cysteinyl-L-glycine

Seven g Thiopropyl-Sepharose 6B (Pharmacia Fine Chemicals) is swollen in 2 liters 10 mM phosphate buffer, pH 7.0, for 30 min. The liquid is sucked off on a glass filter funnel. The protecting groups of the Thiopropyl-Sepharose are removed by suspending the gel in 100 ml 0.3 M sodium bicarbonate, containing 1% dithiothreitol and 1 mM disodium EDTA, adjusted to pH 8.4. The suspension is allowed to stand for one hour. The gel then washed with 110.1 M acetic acid containing 0.5 M sodium chloride, and 1 mM disodium EDTA. The liquid is gently sucked off. The washing is repeated several times. The gel is suspended in 60 ml 0.1 M phosphate buffer and 100 mg cystinyl-diglycine (Feinbiochemica Heidelberg) is added. The suspension is allowed to stand for one hour, at 37°C. The gel is sucked off and the filtrate containing reduced cysteinylglycine is used for the synthesis of cysteinylglycinedopa. To the filtrate are added 35 mg L-dopa, and 40 mg mushroom tyrosinase (Sigma 2430 U/mg).

Preparation of cysteinylglycinedopa

The formation of products is followed by UV-absorption and HPLC as described in the papers dealing with the preparation of cysteinyl-dopas (22, 23). The incubation is stopped by addition of 6 ml 4 M perchloric acid, when the ratio for the UV-absorption at 255 nm and 292 nm is 0.7. The products are purified on a semipreparative column (7.8 mm × 30 cm) packed with 10 Microbondapack C¹⁸ (Waters Associates). As mobile phase an aqueous solution containing 6 g methanesulphonic acid and 3 g phosphoric acid per litre was used and the pH was adjusted to 3.0. The flowrate was 2 ml/min. The effluent was monitored with a UV-detector working at 290 nm. The effluents from the semipreparative column containing a compound with the same relative UV-absorption maxima and minima as 5-S-cysteinyl-dopa were assumed to contain 5-S-cysteinylglycinedopa, and were further processed. The effluents were combined and freed from the mobile phase by absorption of the amino acid onto a cation exchange resin column, 0.5 × 5 cm, containing Dowex 50W-X4, (H⁺-form). The column was washed twice with distilled water, and then eluted with 1 M HCl.

The fractions containing material with UV-absorption maxima at 255 and 292 nm were collected.

The identity of the isolated product as 5-S-cysteinylglycinedopa was established by

(a) Proton NMR analysis in D₂O, recorded on a Varian XL 300 NMR Spectrometer. The spectrum showed a pattern characteristic of two aromatic protons in meta-position. The spectrum was also consistent with two methine groups and three methylene groups. Thus the spectrum was in accordance with the structure of 5-S-cysteinylglycinedopa.

(b) Determination of the products after acid hydrolysis with 6 M HCl, containing 1% 2-mercaptoethanol at 120°C for 24 hours. Amino acid analysis of the hydrolysate yielded 77% of the theoretically expected amount of L-glycine. 5-S-Cysteinyl-dopa was demonstrated by HPLC after gentle hydrolysis in 1 M HCl, but in lower amounts.

(c) UV-spectrum, which showed maxima at 292 and 255 nm with a ratio of 0.8.

Molar absorption of 5-S-cysteinylglycinedopa was calculated to be 2600 at 292 nm on the basis of sulphur analysis of a defined quantity of the substance.

5-S-glutathionyl-dopa was prepared in a similar way as 5-S-cysteinylglycinedopa. NMR data,

Table I. Dopa, 2-S-CD, 5-S-CD, 5-S-cysteinylglycinedopa(5-S-CGD) and 5-S-glutathionyl-dopa(5-S-GD) in cells and media from cultured human melanoma cells, IGR 1

Values in nmole/g cells, or nmole/ml media

Exp no.	Dopa	2-S-CD	5-S-CD	5-S-CGD	5-S-GD	2-S-CD/5-S-CD
<i>Cells</i>						
1	2.0	44	187	0.13	1.3	0.24
2	3.1	70	304	0.38	4.0	0.23
3	2.3	41	184	0.32	1.2	0.22
<i>Media</i>						
1	1.0	0.63	2.8	0.05	0.68	0.23
2	1.6	1.6	6.6	0.13	0.66	0.24
3	1.7	1.2	5.6	0.11	0.90	0.21

relative absorption at 292 and 255 nm and analysis for glycine and glutamic acid after hydrolysis were consistent with 5-S-glutathionyl-dopa.

γ -Glutamyl-5-S-cysteinyl-dopa was prepared from 5-S-glutathionyl-dopa by hydrolysis in 1 M HCl at 100°C for 1 h. At HPLC analysis in the system described, γ -glutamyl-5-S-cysteinyl-dopa appeared well separated before 5-S-glutathionyl-dopa. Material for analysis was obtained by chromatography on a semipreparative column (7.8 mm \times 30 cm) packed with 10 Microbondapack C¹⁸ (Waters Associates). The mobile phase was eliminated as described for preparation of cysteinylglycinedopa. Proton NMR analysis was consistent with γ -glutamyl-5-S-cysteinyl-dopa.

2-S-Glutathionyl-dopa was not isolated but a 30 min incubate of dopa (1 mM), glutathione (3 mM), and mushroom tyrosinase was investigated for the ratio between 5-S-glutathionyl-dopa and the other major product, assumed to be 2-S-glutathionyl-dopa. This compound had the same relative UV-absorption with maxima at 292 and 255 nm and minima at 271 and 246 nm as 2-S-cysteinyl-dopa with a ratio of absorption at 292 and 255 nm of 1.4.

We were interested to find out if glutathione added to dopaquinone in the same ratio as cysteine at the 2- and 5-positions. A Spectra Physics SP 4270 integrator with Varian, Vario-Chrom UV-detector was used in the analysis of this problem. The relevant areas of the absorption curves of 2-S and 5-S-cysteinyl-dopa were compared with those of the corresponding glutathionyl-dopas.

Quantitative analyses of dopa, 2-S-cysteinyl-dopa, 5-S-cysteinyl-dopa, 5-S-cysteinylglycinedopa and 5-S-glutathionyl-dopa were performed by HPLC with electrochemical detection as previously described (23). As mobile phase an aqueous solution containing 6 g methanesulphonic acid and 3 g phosphoric acid per litre at pH 3.0 was used.

Cultures of a pigment producing human melanoma cell line (IGR 1) were obtained from Dr Christian Aubert, Marseille, and have been kept since March, 1982, in culture at the Tornblad Institute, University of Lund, by methods previously described (16). The medium used was minimal essential medium (MEM) + 10% fetal calf serum (Flow). On the third day of passage, the cells, about 200 mg, were harvested, homogenized in an Ultraturax homogenizer after addition of 5 ml 0.4 M perchloric acid. After centrifugation the supernatants were transferred to tubes containing 200 mg Al₂O₃, 200 mg EDTA, and 10 mg sodium metabisulfite and the pH was adjusted to 7.5 with 2 M sodium carbonate. The alumina was spun down at 4000 r.p.m. and washed twice with distilled water before elution with 1 ml 0.2 M HCl. Media was purified on alumina in the same manner, after addition of 1 part 4 M perchloric acid to 9 parts medium, and eluates from both preparations were analysed with HPLC and electrochemical detection. A mixture of the compounds synthesized as above was used as external standards.

RESULTS

In homogenates of melanoma cells and in media, dopa, cysteinyl-dopas, cysteinylglycine-dopa and glutathionyl-dopas were found. The amounts are given in Table I. The ratio between the 2-S-cysteinyl-dopa(2-S-CD) and 5-S-cysteinyl-dopa(5-S-CD) isomers was simi-

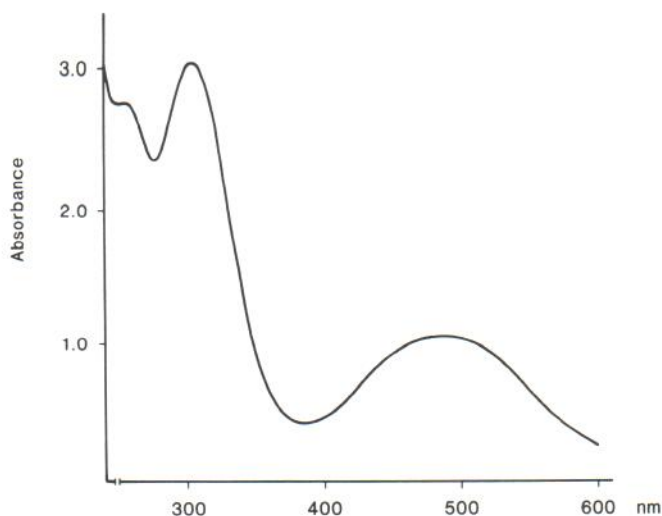


Fig. 1. Absorption spectrum of the red compound formed when incubating 0.71 μ mole 5-S-gluthionyldopa with 2.8 μ mole sodium meta periodate for 5 min at 4°C.

lar in all three experiments, 0.23 (range 0.21–0.24). The relative amounts of the catecholic compounds examined, in the cells, and in the medium, are shown in Table II. γ -Glutamyl-5-S-cysteinyl-dopa was not detected in the cell extracts or in the media.

Integrator analysis of the areas of UV-absorption curves of 2-S- and 5-S-cysteinyl-dopa and of 2-S- and 5-S-gluthionyldopa after incubation of dopa + tyrosinase with cysteine and glutathione, respectively, showed the same ratio between 2-S- and 5-S-cysteinyl-dopa as between 2-S- and 5-S-gluthionyldopa. Thus the ratio between the UV-absorption area of 2-S-cysteinyl-dopa and 5-S-cysteinyl-dopa at 255 nm was 0.135 and between 2-S-gluthionyldopa and 5-S-gluthionyldopa 0.139. The corresponding ratios at 292 nm were 0.211 and 0.215.

In the course of the work with isolated 5-S-gluthionyldopa we observed a strong tendency of this compound to form black pigment when exposed to cells or media in neutral solutions. An experiment was performed in order to get preliminary information on the nature of the pigment formed from 5-S-gluthionyldopa.

0.71 μ mole 5-S-gluthionyldopa was mixed with 2.8 μ mole sodium meta periodate in 2 ml 0.1 M sodium acetate buffer, pH 4.8 at 4°C for 5 min.

At mixing of 5-S-gluthionyldopa with the oxidizing compound, a red colour with a tinge of violet appeared instantly. After a few seconds a warm red colour developed, which remained throughout the experiment. The absorption spectrum after 5 min is shown in Fig. 1. The absorption of the colour had a broad maximum at 480 nm. After some more time had elapsed a darkening of the colour was observed and after 30 min the mixture had become totally black.

Table II. Relative molar amounts of catechols in homogenates from cultured melanoma cells and in culture media

Mean of three experiments. Concentration of dopa in cells = 1

	Dopa	2-S-CD	5-S-CD	5-S-CGD	5-S-GD
Cells	1	20	90	0.1	0.8
Media	0.6	0.5	2	0.04	0.3

DISCUSSION

Evidence for the presence of glutathionyl-dopa in melanoma cells has previously been produced (9), but the present investigation provides quantitative data on a well established and thoroughly studied melanoma cell line. In addition a new dopa compound related to glutathione-metabolism has been demonstrated for the first time in melanoma cells, namely 5-S-cysteinylglycinedopa. This substance may appear as a product of nucleophilic addition of cysteinylglycine to dopaquinone but it is also possible that 5-S-glutathionyl-dopa is hydrolysed by a γ -glutamyltranspeptidase, which reaction has been demonstrated in melanoma tissue.

The incubation experiments with integrator analysis of UV-absorption curves at 255 and 292 nm of the 2-S- and 5-S- addition products of cysteine and glutathione to dopaquinone showed identical ratios for the cysteinyl- and glutathionyl-isomers at the different wavelengths. Ito et al. (24) have previously reported ratios between 2-S- and 5-S-glutathionyl-dopa that were close to those of 2-S- and 5-S-cysteinyl-dopa.

The observations on the pigment formation from 5-S-glutathionyl-dopa are remarkable since the peptide bond of cysteine to glutamic acid prevents the reaction necessary for the formation of pheomelanin. The occurrence of hydrolytic enzymes in our media was too low to explain the pigment formation from 5-S-glutathionyl-dopa as a result of splitting the peptide bond. The pigment formed from 5-S-glutathionyl-dopa was black and not brown as pheomelanins. Furthermore the chromophore formed by periodate oxidation of 5-S-glutathionyl-dopa had a broad maximum at 480 nm, similar to dopachrome.

From Table II it is evident that the cysteinyl-dopas are the dominant catecholic compounds in the cells. The molar concentration is roughly a hundred times that of dopa. The concentrations of dopa and glutathionyl-dopa are of the same order, while the concentration of cysteinylglycinedopa is one order of magnitude lower.

The low quantity of dopa compared with cysteinyl-dopa in the melanocytes may reflect the fact that dopa in contrast to cysteinyl-dopa acts as a cosubstrate of tyrosinase and therefore is consumed in the presence of this enzyme (19). The much higher quantities of cysteinyl-dopa than of glutathionyl-dopa and of cysteinylglycinedopa are remarkable since glutathione is considered to be the dominant thiol in the cells (25). The lesser amount of glutathionyl-dopa than of cysteinyl-dopa may reflect a rapid catabolism of glutathionyl-dopa but may also be explained by dopa oxidation in a compartment with a higher concentration of cysteine than of glutathione.

γ -Glutamylcysteine is an intermediate in the synthesis of glutathione (25). The absence of γ -glutamyl-5-S-cysteinyl-dopa in our cells indicates that glutathione is not formed at the site of dopa oxidation, or is formed at a very low rate. It seems unlikely that γ -glutamyl-5-S-cysteinyl-dopa should be metabolized more rapidly than other thiol addition products of dopaquinone.

The relatively high quantities of the different catecholic compounds in the media may be due to release from living or dead cells, but the presence of tyrosinase in the medium (18) may also explain the levels of catecholic compounds observed. Information on thiol concentrations in the cells and in medium will be necessary for calculations on to what extent cysteinyl-dopa and glutathionyl-dopa in medium are formed in cells or in medium.

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