Oral Oncology 46 (2010) 42–48

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Chemoprevention of 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis in hamster cheek pouch by topical application of resveratrol complexed with 2-hydroxypropyl-β-cyclodextrin

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ARTICLE INFO

Article history:
Received 25 August 2009
Received in revised form 21 October 2009
Accepted 21 October 2009
Available online 20 November 2009

Keywords:
Oral cancer
Preneoplastic lesions
Resveratrol
Cyclodextrins
Chemoprevention
Hamster cheek pouch model
DMBA

SUMMARY

Oral squamous cell carcinoma (OSCC) develops slowly and it is usually preceded by identifiable oral preneoplastic lesions (OPLs): chemoprevention could be a promising approach. Resveratrol (RV) is a plant-based agent characterized by a strong *in vitro* antineoplastic action, but this effect has not been clinically confirmed owing to its metabolic inactivation. In order to circumvent this limitation and to improve RV efficacy, it was locally applied and complexed with a protective and solubilising vehicle (2-hydroxypropyl-β-cyclodextrin, HPβCD). The experimentation was performed *in vitro* on 7,12-dimethylbenz[a]anthracene-induced hamster OSCC cell line (HCPC I) and *in vivo* in the related animal model, by comparison of two RV–HPβCD formulations (cream and mouthwash) and RV alone. Vehicles and RV-formulations were free from toxicity. Antiproliferative action of RV on HCPC I was concentration- and time-dependent, and was improved in HPβCD-formulations. *In vivo,* RV prevented OPL and OSCC appearance and growth. Here, too, HPβCD-formulations (mainly mouthwash) demonstrated the best chemopreventive effects in terms of lesions prevalence, multiplicity, dimension, and histological signs of malignancy. HPLC detection of RV corroborated that its action is concentration-correlated and is improved by its inclusion in HPβCDs. In summary, our study demonstrates that RV is effective in the chemoprevention of DMBA-induced oral carcinogenesis and when it is complexed with HPβCDs its efficacy is significantly improved.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most prevalent and refractory cancers worldwide.[1] Typically it develops slowly and cumulatively after repeated insults of carcinogens (such as tobacco and alcohol). It is frequently preceded by clinically identifiable alterations of the oral mucosa called oral premalignant lesions (OPLs), mainly in the form of leukoplakias.[2] Despite their high incidence worldwide,[3] OPLs cannot be effectively treated.[4,5] This multi-step OSCC development provides the rationale for its chemoprevention.

Resveratrol (RV, 3,4′,5-trihydroxy-trans-stilbene), a phytoalexin produced by plants in response to attacks by parasites or to stress, has been extensively studied on account of its antineoplastic action on many tumour cell lines.[6,7] RV has been administered systemically for *in vivo* trials,[7,8] though the results have been disappointing owing to its low hydrosolubility and unsatisfactory pharmacokinetics.[9] In fact, it is metabolized and inactivated very extensively and quickly.[10–12] Due to the low metabolism of RV in epithelial tissue,[13] its topical administration on oral mucosa may be an attractive way to circumvent its systemic degradation.

Cyclodextrins are oligosaccharide carriers[14,15] widely investigated as drug action enhancers,[16,17] including topically applied molecules.[18,19] 2-Hydroxypropyl-β-cyclodextrin (HPβCD), an hydrophilic semisynthetic derivative, is one of the most commonly utilized due to its hydrosolubility and complexation potential, especially towards heterocycles and aromatics.[14,15]

This preclinical study set out to determine whether: (i) topical application of RV can prevent development of OPLs and OSCCs, (ii) HPβCD complexes (dispersed in an oil/water emulsion to obtain a cream or dissolved in a solution to mimic a mouthwash) improve RV activity.

The study was performed both *in vitro* on the 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster OSCC cell line (HCPC I)[20] and *in vivo* in the related animal model (DMBA-induced OSCC in Syrian hamster cheek pouches).[21–23]

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doi:10.1016/j.oraloncology.2009.10.007

Materials and methods

Vehicles

We used as vehicles ethanol, mouthwash and cream. To obtain mouthwash, 30% (250 mM) Kleptose®HPB (kind gift from Roquette, Lestrem, France), 0.2% Kemipur® (A.C.E.F., Fiorenzuola d'Arda, Italy), 0.3% phenoxyparaben (Sinerga, Milano, Italy) were added to saline solution. For cream, 7% Finsolv®TN (A.C.E.F.) and then 2% Sepigel®305 (Seppic, Paris, France) were admixed with mouthwash and homogenized.

Formulations containing RV

The formulations tested were RV-ethanol, RV-cream and RV-mouthwash. RV-ethanol was the saturated solution of RV in ethanol (285 mM). RV-mouthwash and RV-cream were obtained as follows: 100 mM RV (Cabru, Arcore, Italy) was dispersed in saline solution previously supplemented with HPβCD. The chemicals used for mouthwash and cream were then added. All complexes were characterized and RV loading was assessed as previously described.[24] The final RV concentration detected of both HPβCD-formulations was 74.5 mM.

Cell lines

The HCPC I cell line[20] (kindly provided by Professor D.T. Wong, UCLA, USA) was cultured in 75 cm^2 flasks (TPP AG, Trasadingen, Switzerland) in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin G, 40 μg/ml gentamicin sulphate and 2.5 μg/ml amphotericin B at 37 °C in a humidified 5% CO_2 atmosphere (unless otherwise specified, reagents were from Sigma–Aldrich, Milano, Italy).

Cytotoxicity assay

Cells were seeded at a density of 5×10^2 per well in 96-well plates (TPP AG). After one day, the medium was replaced and supplemented with serial concentrations of RV from 5 μM to 100 μM, dissolved in ethanol (RV-ethanol) or complexed with HPβCD (RV-cream or RV-mouthwash). Equivalent amounts of vehicles alone were used as the negative controls. After 24, 48 and 72 h of incubation, cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)-assay[25] and expressed as average percentage of absorbance in treated cells versus control (culture medium alone).

Animals

The right cheek pouches of 90 Syrian golden hamsters (Charles River, Calco, Italy) were painted every Monday, Wednesday and Friday with 0.5% DMBA dissolved in paraffin oil, using a No. 4 paintbrush.[21,22] Animals were randomly divided into seven groups: three groups treated with RV dissolved in ethanol (n = 15, RV-ethanol-group) or with RV complexed with HPβCD formulated in cream (n = 15, RV-cream-group) or mouthwash (n = 15, RV-mouthwash-group) respectively; three corresponding control groups (each n = 10) receiving the vehicles; one group painted with DMBA only (n = 15, DMBA-group).

Every Tuesday and Thursday, all formulations were topically applied in situ for 5 s. The RV-ethanol-group received 35 μl of RV-ethanol. RV-mouthwash and RV-cream contained the same amount of RV in the minimum volume needed for easy administration (140 and 250 μl, respectively); the cream was administered with a paintbrush, whereas the mouthwash was applied by a syringe with a plastic spout and the pouches were shaken to mimic a mouth rinse. The three control groups were treated in the same way with the corresponding vehicles alone.

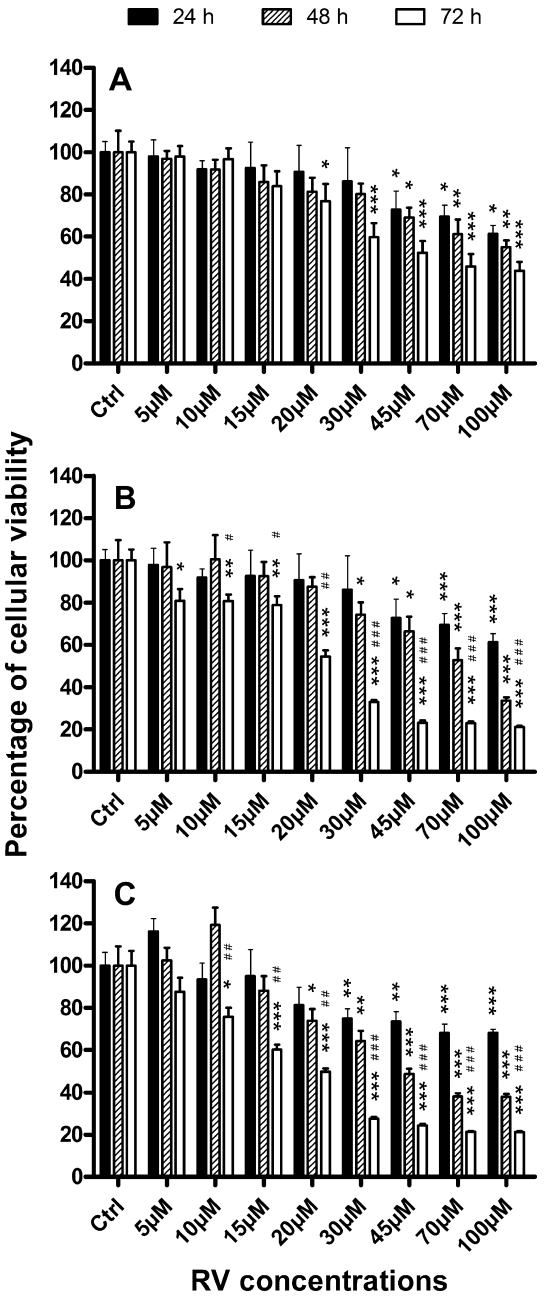

Figure 1 Antiproliferative action of RV on HCPC I is concentration- and time-dependent and improved by HPβCD inclusion. For RV-ethanol (A), 50% of cell growth inhibition was achieved only after 72 h at 45 μm, while for RV-cream (B) it was reached after 48 and 72 h at 70 and 20 μm, and for RV-mouthwash (C) at 45 and 20 μm respectively. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus control (medium alone). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ versus RV-ethanol (at the same time and concentration).

From the 3rd week, animals were weighed and lightly anesthetized weekly. Pouches were everted and lesions were counted, measured, scored and photographed. The end-points for data analysis included prevalence, multiplicity, average lesion diameter, and a "pathological score" (PS) to express the overall situation of a cheek pouch as a number. Lesions were arbitrarily scored in terms of size and severity: 0 for no lesions, 1 for each OPL, 2 for an exophytic lesion (ExL) <1 mm in diameter, 3–8 for each ExL bigger