

RESEARCH ARTICLE

Chemopreventive potential of 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one on 7,12-dimethylbenz[a]anthracene (DMBA) induced hamster buccal pouch carcinogenesis

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Abstract

In the present work, a new *bis* heterocyclic compound comprising both the piperidone and thiohydantoin nuclei namely 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one was synthesised and characterised with the help of mp, elemental analysis, FT-IR, MS and one-dimensional NMR (¹H and ¹³C) spectra. The inhibitory effect of 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one on 7,12-dimethylbenz[a]anthracene (DMBA) induced buccal pouch carcinogenesis was investigated in Syrian male hamsters. All the hamsters that were painted with DMBA on their buccal pouches for 14 weeks developed squamous cell carcinoma. Administration of 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one effectively suppressed the oral carcinogenesis initiated with the DMBA as revealed by a reduced incidence of neoplasms. Lipid peroxidation, glutathione (GSH) content and the activities of glutathione peroxidase (GPx), glutathione S-transferase (GST) were used to biomonitor the chemopreventive potential of 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one. Lipid peroxidation was found to be significantly decreased, whereas GSH, GPx, GST and GGT were elevated in the oral mucosa of tumour bearing animals. Our data suggest that 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one may exert its chemopreventive effects in the oral mucosa by modulation of lipid peroxidation, antioxidants and detoxification systems.

Keywords: Oral cancer; 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one; chemoprevention; lipid peroxidation; hamster buccal pouch carcinogenesis

Introduction

The sulphur analogues of hydantoins with one or both carbonyl groups replaced by thiocarbonyl groups are known as thiohydantoins. There has been much interest in the synthesis and properties of 2-thiohydantoin derivatives as useful synthetic intermediates for a wide range of applications [1,2] as hypolipidaemic, anticarcinogenic, antimutagenic, antithyroidal, antiviral (e.g. against herpes simplex virus, HSV, human immunodeficiency virus (HIV), tuberculosis, antimicrobial (antifungal and antibacterial), anti-ulcer and anti-inflammatory agents, as well as pesticides.

Among the nitrogen containing heterocyclic compounds, piperidones have gained considerable importance owing to their varied biological properties and therapeutic importance. Baliah et al. reviewed the importance of piperidin-4-ones as intermediates in the synthesis of several physiologically active compounds [3,4]. In corollary of their interesting biological and pharmaceutical properties and synthetic utility, substantial interest has been demonstrated towards piperidones; this substructure is widely present in numerous alkaloids and synthetically derived molecules of biological importance [5].

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(Received 13 December 2009; revised 09 February 2010; accepted 12 February 2010)

ISSN 1475-6366 print/ISSN 1475-6374 online © 2010 Informa UK, Ltd.
DOI: 10.3109/14756361003724786

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Squamous cell carcinoma of the oral cavity is the sixth most common malignant neoplasm worldwide and constitutes 47% of all cancers in the Indian subcontinent [6]. Despite advances in cancer detection and therapy, the mortality rate of oral cancer remains high and the 5 year survival rate is among the lowest of the major cancers [7]. Patients with oral squamous cell carcinoma are susceptible to multiple primary and secondary tumours due to the phenomenon of 'field cancerisation' [8,9]. Furthermore, treatment of these tumours often results in severe disfigurement and functional compromise.

Chemoprevention offers a novel approach to control the incidence of oral cancer. The buccal mucosa of the Syrian hamster is an excellent model for the investigation of oral cancer development and intervention by chemopreventive agents. Squamous cell carcinomas induced by the application of 7,12-dimethylbenz[a]anthracene (DMBA) to the cheek pouch of the Syrian hamsters are morphologically and histologically similar to human tumours [10,11]. In addition, hamster tumours express many metabolic and molecular markers that are expressed in human oral cancer [12–14].

Several markers have been developed to biomonitor chemoprevention. These are based on the fact that chemopreventive agents can exert their anticarcinogenic effects by one or a combination of the following mechanisms: inhibiting formation of reactive carcinogenic metabolites, induction of enzymes that detoxify carcinogens, scavenging reactive oxygen species, influencing apoptosis and inhibiting cell proliferation [15,16]. The tripeptide glutathione, a physiologically important nucleophile and the enzymes glutathione peroxidase (GPx), glutathione S-transferase (GST) and gamma-glutamyltranspeptidase (GGT), which utilise reduced glutathione (GSH) as their substrate are significant as biomarkers of chemoprevention owing to their antioxidant and detoxification properties [17–19].

A large number of chemopreventive agents have been identified in epidemiological and experimental studies, pre-clinical systems and clinical observations [20,21]. However, the toxic side effects produced by some of these agents have limited their extensive use [22]. There is therefore a need to identify synthetic compounds that have significant chemopreventive potential. In view of our continued interest in the development of simpler and more convenient synthetic routes for achieving the biologically challenging hybrid heterocyclic systems [23–31] and as part of our ongoing research programme, we have designed a system, which combines both bioactive thiohydantoin and piperidone nuclei together to give a compact structure like the title compound, 3-(2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylidene)-2-thioxoimidazolidin-4-one and we investigated the effects of 3-(2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylidene)-2-thioxoimidazolidin-4-one in a hamster buccal pouch carcinogenesis model using lipid peroxidation, GSH, GP_x, GST and GGT as biochemical end points of chemoprevention.

Materials and methods

Chemicals

General remarks

We used thin layer chromatography (TLC) to assess both the reactions and the purity of the products. The reported melting point was taken in open capillaries and was uncorrected. IR spectra were recorded in KBr (pellet forms) on a Thermo Nicolet-Avatar-330 FT-IR spectrophotometer (Thermo Fisher Scientific Inc, Waltham, USA) and only note worthy absorption values (cm⁻¹) were listed. One dimensional ¹H and ¹³C NMR spectra were recorded at 400 MHz and 100 MHz respectively on a Bruker AMX 400 NMR spectrometer (Bruker Biospin International, Ag, Aegerstrasse, Switzerland) using DMSO-*d* as solvent. The electron spray impact (ESI) positive (+ve) mass (MS) spectrum was recorded on a Bruker Daltonics LC-MS spectrometer. Satisfactory microanalysis was obtained on a Carlo Erba 1106 CHN analyser (Fisher Scientific Inc, Waltham, USA). All the chemicals were purchased from Fluka, Sigma-Aldrich chemicals (St. Louis, USA) and Spectrochem Pvt. Ltd., (Mumbai, India).

The parent compounds 2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-one and 2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-one thiosemicarbazone were prepared according to the literature [3,4].

Typical procedure for the synthesis of 2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-one

A mixture of ammonium acetate (0.1mol), 4-fluorobenzaldehyde (0.2mol) and butan-2-one (0.1mol) were dissolved in 95% alcohol (80 mL) and the solution was heated on a hot plate with gentle swirling until the colour of the mixture changed to orange. The mixture was cooled and poured into diethyl ether (100 mL) and concentrated hydrochloric acid (14 mL) was added. The precipitated hydrochloride was collected by filtration and recrystallised from the ethanol-ether mixture. The hydrochloride was dispersed in acetone and concentrated aqueous ammonia was added dropwise until a clear solution was obtained. The clear solution was poured into cold water and the solid precipitation was collected and recrystallised from ethanol.

Typical procedure for the synthesis of 2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-one thiosemicarbazone

A mixture of 2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-one (0.01mol) and thiosemicarbazide (0.01 mol) in ethanol (60 mL) was refluxed for two hours on a steam bath, cooled, then the separated solid was filtered and washed with water. The solid was subjected to column chromatography using benzene:chloroform (1:1) as eluent to afford the 2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-one thiosemicarbazone.

Typical procedure for the synthesis of novel new 3-(2,6-bis(4-fluorophenyl)-3-methylpiperin-4-ylidene)-2-thioxoimidazolidin-4-one

To a well stirred solution of 2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-one thiosemicarbazone (0.01mmol) and anhydrous sodium acetate (0.01 mol) in 30 mL of ethanol, was added chloroethyl acetate (0.01 mmol in 15 mL of ethanol) drop wise through the addition funnel for about 10 min. Then the reaction mixture was refluxed further for 4 h. After completion of the reaction, the reaction mixture was poured into ice cold water and the solid mass was collected and recrystallised twice from the ethanol to give the title compound with a yield of 78% at a mp of 182°C.

Spectral data of 3-(2,6-bis(4-fluorophenyl)-3-methylpiperin-4-ylidene)-2-thioxoimidazolidin-4-one

IR (KBr) (cm⁻¹): 3412, 3304, 3071, 3030, 2985, 2931, 2869, 1717, 1635, 1597, 1221, 1030, 535, 836, 766; MS: *m/z* = 415, [M+1]⁺. Molecular formula C₂₁H₂₀F₂N₄OS; Elemental analysis: Carbon 60.85_{cal.} (60.77_{found.}), Hydrogen 4.86_{cal.} (4.75_{found.}), Nitrogen 13.52_{cal.} (13.44_{found.}); ¹H NMR (δ ppm): 0.78–0.8 (*d*, 3H, CH₃ at C-3, *J*_{CH3,3a} = 6.58 Hz), 2.07–2.09 (*m*, 1H, H_{5a}), 2.54–2.59 (*m*, 1H, H_{3a}), the signal for 1H, NH of piperidine merged with water peak, 2.67–2.79 (*m*, 1H, H_{5e}), 3.46–3.48 (*d*, 1H, H_{2a'}, *J*_{2a,3a} = 10.61 Hz), 3.54–3.58 (*dd*, 1H, H_{6a'}, *J*_{6a,5e} = 2.46 Hz, *J*_{6a,5a} = 12.46 Hz), 3.78 (*s*, 2H, CH₂ of imidazolidine), 7.12–7.51 (*m*, 8H, Ar-H's), 11.72 (*s*, NH of imidazolidine); ¹³C NMR (δ ppm): 11.8 -CH₃ at C-3, 32.5 C-5, 37.2 C-3, 44.5 CH₂ of imidazolidine, 61 C-6, 67.7 C-2, 114.6–160.7 Ar-C's, 162.4, 162.5, *ipso*-C, 163.4 C=N, 169.2 C=O, 173.9 C=S.

Animals

Male Syrian hamsters aged 6–10 weeks and weighing between 90–110 g were obtained from the Central Animal House, Annamalai University, India, for use in this study. The animals were housed six to a polypropylene cage and provided with food and water *ad libitum*. The animals were maintained in a controlled environment under standard conditions of temperature and humidity with an alternating 12 h light:dark cycle. All animals were fed with a standard pellet diet (Mysore Snack Feed Ltd., Mysore, India).

Treatment Schedule

The animals were randomised into experimental and control groups and divided into four groups of six animals each. Animals in group 1 were painted with a 0.5% solution of DMBA in liquid paraffin on their right buccal pouches using a number 4 brush, three times per week for 14 weeks, with each application leaving approximately 0.4 mg DMBA [32]. Group 2 animals were painted with DMBA as in group 1 but with the addition of the oral administration of the title compound, 3-(2,6-bis(4-fluorophenyl)-3-methylpiperin-4-ylidene)-2-thioxoimidazolidin-4-one at a concentration of 1 mg/kg body weight, three times per week and on alternate days to the DMBA application. Animals in group 3 received only 3-(2,6-bis(4-fluorophenyl)-3-methylpiperin-4-ylidene)-2-thioxoimidazolidin-4-one as in group 2. Group 4 were the

untreated control animals received neither DMBA nor 3-(2,6-bis(4-fluorophenyl)-3-methylpiperin-4-ylidene)-2-thioxoimidazolidin-4-one. The experiment was terminated at 14 weeks and all animals were sacrificed by cervical dislocation after an overnight fast. Fresh tissue was used for estimations.

Estimations

The thiobarbituric acid reactive substances (TBARS) released from the endogenous lipid peroxides reflecting the lipid peroxidation process were assayed in tissues as described by Ohkawa et al. [33]. Superoxide dismutase activity was assayed by the method of Oberley et al. [34], based on the inhibition of the formation of NADPH phenazine methosulphate and nitroblue tetrazolium. Catalase was assayed colorimetrically by the method of Sinha. [35]. Reduced GSH was determined using the method of Beutler and Kelley [36]. GPx activity was assayed by following the utilisation of hydrogen peroxide according to the method of Rotruck et al. [37]. The activity of GST was assayed using the method of Habig et al. [38] using 1-chloro, 2,4-dinitrobenzene (CDNB) as the substrate. Tissue protein was estimated by the method of Lowry et al. [39] using bovine serum albumin as the standard.

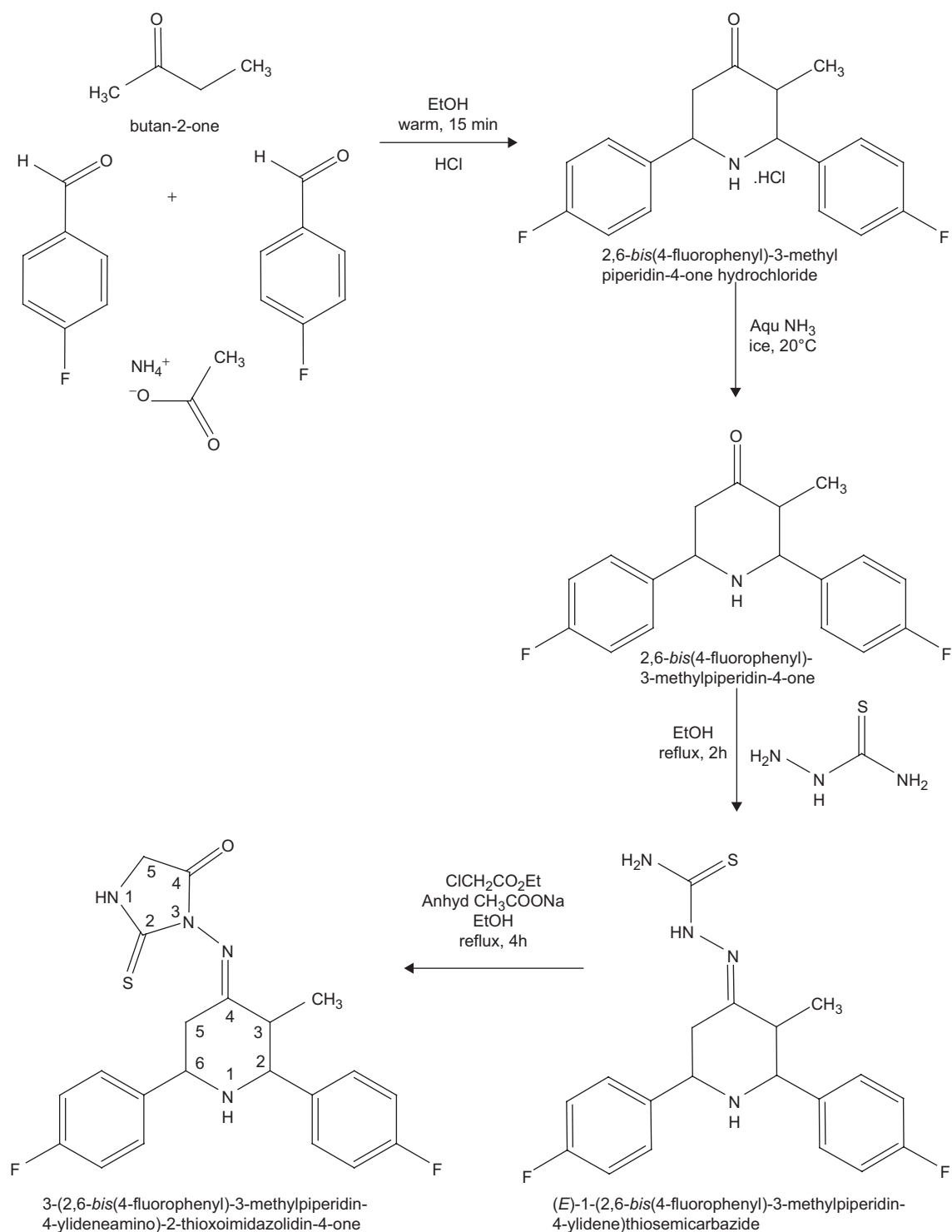
Statistical analysis

The data have been expressed as mean ± standard deviation (SD). Statistical analysis on the data for body weights and tumour burden was carried out using Student's *t* test. Tumour incidence was statistically compared using χ^2 -test. Statistical analysis on the data for biochemical assays was analysed using analysis of variance (ANOVA) and the group means were compared by the least significant difference test (LSD). The results were considered statistically significant if *p* < 0.05.

Results and discussion

In the present work, a new series of *bis* heterocycles comprising both piperidine and thiohydantoin nuclei namely 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one were synthesised by the treatment of the respective thiosemicarbazones with chloroethyl acetate and anhydrous sodium acetate in refluxing ethanol for 4 h. The synthetic route for the formation of title compound is given in Scheme 1 and a reaction mechanism is shown in Scheme 2. The structure of the synthesised compound is characterised with the help of mp, elemental analysis, FT-IR, MS, one-dimensional NMR (¹H, ¹³C) spectra.

The body weights, tumour incidence, tumour multiplicity, tumour burden, incidence of preneoplastic and neoplastic lesions in the control and experimental animals are shown in Table 1. The mean final body weights were significantly decreased in group 1 compared to the controls (group 4). No significant differences in body weight were observed in groups 2 and 3. In the DMBA painted animals (group 1), the incidence of squamous cell carcinoma (SCC) was 100% with a tumour multiplicity of 1.17

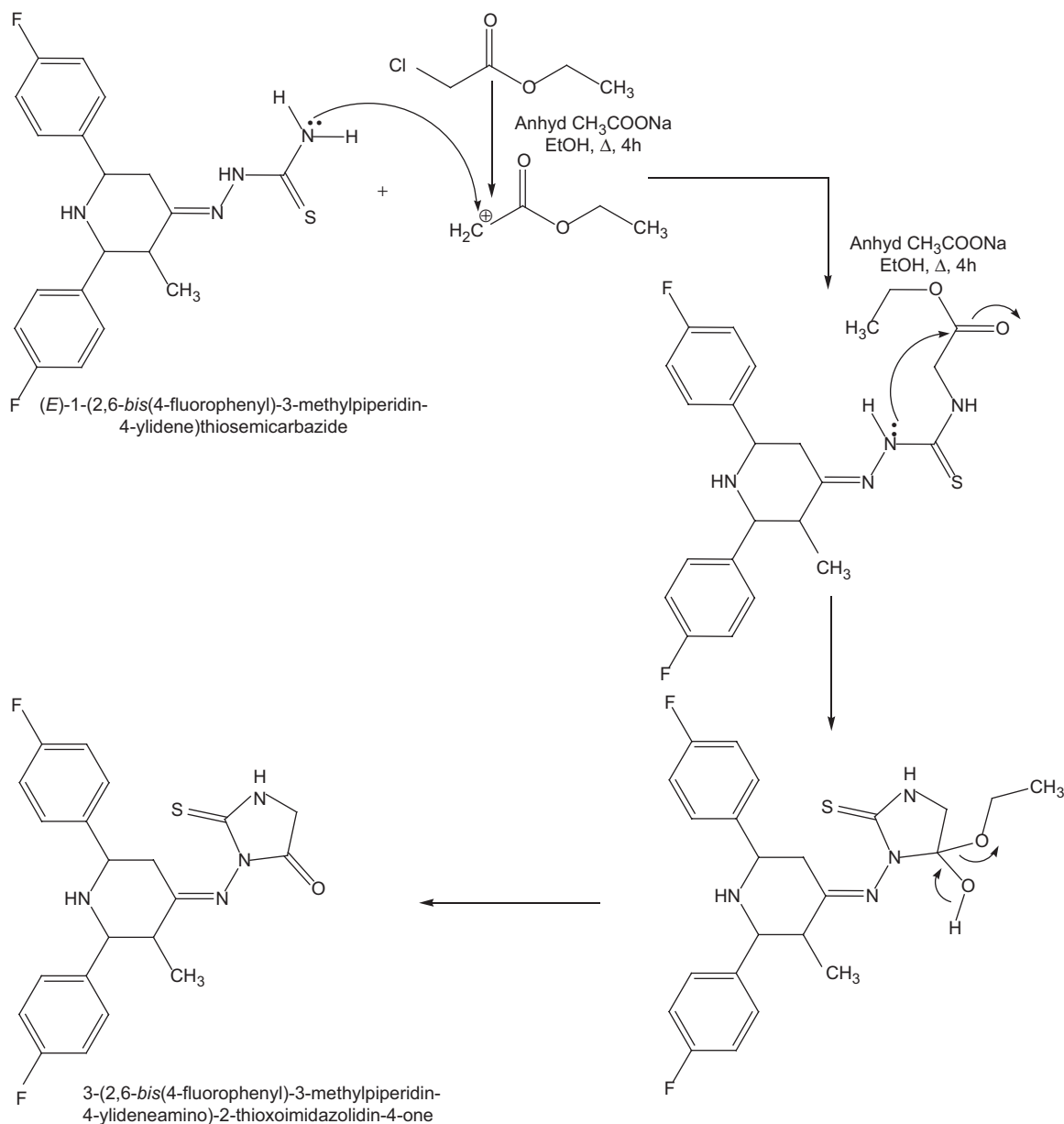


Scheme 1. Synthetic route for the formation of 3-(2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino)-2-thioxoimidazolidin-4-one

per hamster. These tumours were large and exophytic with a mean tumour burden of 208 mm³. In group 2, only one animal developed SCC, while the others exhibited moderate to severe dysplasia without infiltration. Although no tumours were observed in group 3, histopathological examination of the pouches revealed varying degrees of hyperplasia, hyperkeratosis and dysplasia. While administration of 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one (1 and 10 mg/kg

body weight (BW) decreased the tumour incidence as well as any preneoplastic lesions, the inhibitory effect was more pronounced at 10 mg/kg BW of 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one. In group 4, the epithelium was found to be normal, intact and continuous.

The levels of TBARS and the activities of SOD, catalase, GPx and GST on the buccal pouches of the control and experimental animals are shown in Table 2. The changes in



Scheme 2. Proposed reaction mechanism for the formation of the title compound.

Table 1. Body weight, tumour incidence, tumour multiplicity, tumour burden and incidence of preneoplastic and neoplastic lesions in control and experimental animals (mean ± SD; n = 6).

Group	Treatment	Body weight (g)		Tumour incidence	Tumour multiplicity No. of tumours/ Hamsters	Tumour Burden (mm ³)*	Keratosi	Hyperplasia	Dysplasia
		Initial	Final						
1	DMBA	103 ± 9.21	115 ± 10 ^a	6/6	1.17 ± 0.41	208 ± 43.68	+++	+++	+++
2	DMBA + title compound (1mg/kg BW)	105 ± 8.34	125 ± 11	1/6 ^b	0.16 ± 0.4 ^b	5.55 ± 13.6 ^b	+++	+++	++/+++
3	DMBA + title compound (10mg/kg BW)	106 ± 10	126 ± 9	-	-	-	++	+/+++	+
4	Control	110 ± 11	134 ± 11	-	-	-	++	++	+/+++

+, mild; ++, moderate; +++, Severe; -, no change.

*Mean tumour burden was calculated by multiplying the mean tumour volume ($4/3 \pi r^3$) with the mean number of tumours ($r = 1/2$ tumour diameter in mm).

^aSignificantly different from group 4 by student's *t* test ($p < 0.01$).

^bSignificantly different from group 1 by λ^2 -test ($p < 0.001$).

Table 2. The cellular redox status in the buccal pouches of experimental and control animals (mean \pm SD; n = 10).

Group	Treatment	TBARS (n mol/mg protein)	SOD (U ^a /mg protein)	Catalase (U ^b /mg protein)	GSH (mg/g tissue)	GPX (U ^c /mg protein)	GST (U ^d /mg protein)
1	DMBA	4.6 \pm 0.33	2.34 \pm 0.21	1.18 \pm 0.1	0.18 \pm 0.01	10.65 \pm 0.75	5.7 \pm 0.43
2	DMBA+ TitleCompound (1mg/kg BW)	5.49 \pm 0.38*	2.83 \pm 0.25**	1.37 \pm 0.12*	0.23 \pm 0.02*	11.86 \pm 1.31*	6.43 \pm 0.56*
3	DMBA+ Titlecompound (10mg/kg BW)	5.74 \pm 0.56**	4.3 \pm 0.32***	1.54 \pm 0.15***	0.25 \pm 0.01***	12.53 \pm 1.4**	6.92 \pm 0.63***
4	Control	6 \pm 0.92	5.01 \pm 0.43	2.13 \pm 0.21	0.13 \pm 0.01	7.25 \pm 0.51	3.98 \pm 0.34

Significantly different from group 4 (p < 0.001) ANOVA followed by LSD.

* Significantly different from group 1 (p < 0.05) ANOVA followed by LSD.

** Significantly different from group 1 (p < 0.01) ANOVA followed by LSD.

*** Significantly different from group 1 (p < 0.001) ANOVA followed by LSD.

^a The amount of enzyme required to inhibit 50% NBT reduction.

^b μ moles of H₂O₂ utilised/min.

^c μ moles of GSH utilised/min.

^d μ moles of 1-chloro-2,4-dinitrobenzene-reduced glutathione conjugate formed/min.

the levels of TBARS, GSH and the activities of GPx and GST were significantly increased with a decrease in SOD and catalase activities in the DMBA painted animals (group 1) compared to the control (group 4). The administration of 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one significantly enhanced the lipid peroxidation and the antioxidant activity in the buccal pouches of the group 2 and 3 animals compared to group 1. However, the antioxidant enhancing effects were more significant in group 3 (10 mg/kg BW) compared to the other groups. DMBA, an aryl hydrocarbon, is a potent carcinogen that induces significant histological and biochemical changes during HBP carcinogenesis. In the present study, topical application of DMBA to the cheek pouch for 14 weeks resulted in well-differentiated SCCs with a very high tumour burden.

In the DMBA-induced HBP tumours, a low lipid level was associated with enhanced activities of the GSH-redox cycle antioxidants. Lipid peroxidation, which prolongs the G1 phase of the cell cycle, has been suggested to control cell division. Das have demonstrated that tumour cells are more resistant to lipid peroxidation than normal cells [40]. Several studies have documented decreased lipid peroxidation in rapidly proliferating tumours compared with their normal counterparts [40,41]. The thiol antioxidant GSH, which provides cellular protection against ROS in conjunction with GPx, γ -glutamyl transferase (GGT) and glutathione reductase (GR) is recognised to play a key role in regulating cell proliferation [42]. The overexpression of the GSH- and GSH-dependent enzymes has been reported in a wide range of tumours including oral squamous cell carcinoma (OSCC) [43]. The elevated GSH redox cycle of antioxidants may serve to maintain a reduced environment providing a selective growth advantage for the hamster buccal pouch (HBP) tumours. Our results are in agreement with the hypothesis of Slater et al. [44] that an increase in antioxidant capacity is associated with a decrease in lipid peroxidation. In contrast to GSH-dependent antioxidants, the activities of the antioxidant enzymes SOD and CAT were decreased in the HBP tumours. An increase in the activities of GPx with decreased SOD and CAT has been reported in various

tumours, including the hamster cheek pouch carcinoma cell line HCPC-1 [45–47]. It has been reported that reduced activities of SOD and CAT in fast growing tumor tissues can cause accumulation of the superoxide anion (O₂⁻) and H₂O₂ with deleterious consequences including oxidation of critical sulphhydryl groups and conformational changes in functional proteins as well as DNA strand breaks leading to oxidative stress. Oxidative stress has been implicated in cancer progression [48].

Administration of 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one at two different doses (1 and 10 mg/kg BW) significantly suppressed tumour incidence, multiplicity, and tumour burden in the HBP by modulating the oxidant-antioxidant status. The results of the present study substantiate the anticarcinogenic effects of bioactive piperidones and imidazole derivatives as reported in the literature [5,49]. It was found that the administration of synthetic compounds could reverse the susceptibility to lipid peroxidation while simultaneously increasing the antioxidant status in the buccal pouch. These findings support reports by us and other workers that chemopreventive agents exert an “electrophilic counterattack response” characterised by the elevation of antioxidant enzymes [50]. Out of the two doses used in the present study, the maximum dose of 10 mg/kg BW was more effective in chemoprevention. We have demonstrated that 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one suppresses DMBA-induced oral neoplasms as revealed by the reduced incidence of carcinomas. The present preliminary study suggest that 3-[2,6-bis(4-fluorophenyl)-3-methylpiperidin-4-ylideneamino]-2-thioxoimidazolidin-4-one has potential anticarcinogenic properties in experimental animals and could be a promising candidate for chemoprevention.

Acknowledgements

Authors are thankful to NMR Research Centre, Indian Institute of Science, Bangalore for recording spectra. V. Kanagarajan is grateful to the Council of Scientific and

Industrial Research (CSIR), New Delhi, Republic of India for providing financial support in the form of CSIR-Senior Research Fellowship (SRF) in Organic Chemistry. J. Thanusu is thankful to the Annamalai University authorities for providing University Studentship.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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