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Butachlor induced dissipation of mitochondrial membrane potential, oxidative DNA damage and necrosis in human peripheral blood mononuclear cells

Sourabh Dwivedi[a], Quaiser Saquib[a], Abdulaziz A. Al-Khedhairy[a], Javed Musarrat[a,b,*]

[a] Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia
[b] Department of Agricultural Microbiology, Faculty of Agricultural Sciences, AMU, Aligarh 202002, India

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ABSTRACT

Butachlor is a systemic herbicide widely applied on rice, tea, wheat, beans and other crops; however, it concurrently exerts toxic effects on beneficial organisms like earthworms, aquatic invertebrates and other non-target animals including humans. Owing to the associated risk to humans, this chloroacetanilide class of herbicide was investigated with the aim to assess its potential for the (i) interaction with DNA, (ii) mitochondria membrane damage and DNA strand breaks and (iii) cell cycle arrest and necrosis in butachlor treated human peripheral blood mononuclear (PBMN) cells. Fluorescence quenching data revealed the binding constant ($Ka = 1.2 \times 10^4\ M^{-1}$) and binding capacity ($n = 1.02$) of butachlor with ctDNA. The oxidative potential of butachlor was ascertained based on its capacity of inducing reactive oxygen species (ROS) and substantial amounts of promutagenic 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) adducts in DNA. Also, the discernible butachlor dose-dependent reduction in fluorescence intensity of a cationic dye rhodamine (Rh-123) and increased fluorescence intensity of 2′,7′-dichlorodihydro fluorescein diacetate (DCFH-DA) in treated cells signifies decreased mitochondrial membrane potential ($\Delta\Psi m$) due to intracellular ROS generation. The comet data revealed significantly greater Olive tail moment (OTM) values in butachlor treated PBMN cells vs untreated and DMSO controls. Treatment of cultured PBMN cells for 24 h resulted in significantly increased number of binucleated micronucleated (BNMN) cells with a dose dependent reduction in the nuclear division index (NDI). The flow cytometry analysis of annexin V^-/7-AAD^+ stained cells demonstrated substantial reduction in live population due to complete loss of cell membrane integrity. Overall the data suggested the formation of butachlor–DNA complex, as an initiating event in butachlor-induced DNA damage. The results elucidated the oxidative role of butachlor in intracellular ROS production, and consequent mitochondrial dysfunction, oxidative DNA damage, and chromosomal breakage, which eventually triggers necrosis in human PBMN cells.

1. Introduction

Butachlor [N-(butoxymethyl)-2-chloro-2′,6′-diethyl acetanilide] is a selective pre-emergent systemic herbicide widely used for control of a range of annual grass and broad leaf weeds (Chang, 1971). This class of herbicide inhibits the biosynthesis of lipids, alcohols, fatty acids, proteins, isoprenoids and flavonoids (Ecobichon, 2001; Heydens et al., 2002). It is widely recommended herbicides for rice cultivation, which affects soil reduction processes including acetylene reduction activity (ARA) in flooded rice soils (Jena et al., 1987). The increased application of herbicides on rice, tea, wheat, beans and other crops, reportedly exerts detrimental effects on beneficial organisms like earthworms (Muthukaruppan and Gunasekaran, 2010) and other non-target animals (Kumari et al., 2009). Ecotoxicological studies suggested that butachlor and their metabolites may be harmful to aquatic invertebrates (Ateeq et al., 2002, 2006; Vallotton et al., 2009), microbial communities (Min et al., 2002; Widenfalk et al., 2008) and possibly carcinogenic in animals and humans (Panneerselvam et al., 1999; Geng et al., 2005a,b). Butachlor has been suggested to be mutagenic in primary rat tracheal epithelial cells and Chinese hamster ovarian cells (Wang et al., 1987; Hill et al., 1997), and causes stomach tumors in rats. The mutagenicity and carcinogenicity of butachlor and other chloracetamide herbicides like acetochlor, alachlor and metolachlor have been thoroughly reviewed (Dearfield et al., 1999). Coleman et al. (2000) have determined the metabolism of butachlor to 2-chloro-N-(2,6-diethylphenyl) acetamide (CDEPA) and 2,6-diethylaniline (DEA) both in rat and human livers. The common metabolic activation pathway of chloracetamide compounds leading to the formation

* Corresponding author at: Abdul Rahman Al-Jeraisy Chair for DNA Research, Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia. Tel.: +966 558835658; fax: +966 1 4675768.
E-mail address: musarratj1@yahoo.com (J. Musarrat).

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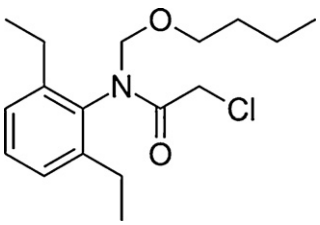

Fig. 1. Chemical structure of butachlor.

of a putative ultimate carcinogenic metabolite, suggests that this class of herbicides have a common mechanism of carcinogenicity. Prolonged exposure to butachlor has also been found to be toxic to spotted snakehead fish (*Channa punctata*), and accumulates through the food chain (Tilak et al., 2007). It has been reported to be neurotoxic to land snails (Rajyalakshmi et al., 1996), genotoxic to toad and frog tadpoles, flounder, and catfish (Ateeq et al., 2005; Geng et al., 2005b; Yin et al., 2007, 2008), and causes DNA strand breaks and chromosomal aberrations in cultured mammalian cells (Sinha et al., 1995; Panneerselvam et al., 1999).

Earlier studies have suggested the possible links between pesticides induced genotoxicity and damage to biological macromolecules in human population exposed to either single or mixture of pesticides (Garaj-Vrhovac and Zeljezic, 2001; Zeljezic and Garaj-Vrhovac, 2002; Padmavathi et al., 2000). Such pesticides when intercalate or covalently bind with DNA molecule may form DNA adducts, which can lead to gene mutations and initiate carcinogenesis, if the adducts are not repaired or misrepaired before DNA replication occurs (Saquib et al., 2010a). Increased DNA damage enhances the probability of mutations occurring in critical target genes and cells, which may trigger the process of carcinogenesis (Eisenbrand et al., 2002). The genotoxicity of butachlor in mammals and invertebrates has been extensively demonstrated (Simpson et al., 1994; Panneerselvam et al., 1999; Geng et al., 2005a,b; Ateeq et al., 2005, 2006). However, no systematic studies have been carried out on the nature and extent of physical interaction of butachlor with DNA, and/or its role as an oxidative genotoxicant in human peripheral blood mononuclear (PBMN) cells. To the best of our understanding, this study provides the first evidence that butachlor as a ligand can bind to DNA with high affinity and have a potential of producing intracellular reactive oxygen species (ROS) leading to oxidative stress, DNA damage and necrotic effects in human PBMN cells. For this investigation, several sensitive techniques such as fluorescence spectroscopy (Zhang et al., 2005; Kashanian et al., 2008; Khan and Musarrat, 2003; Saquib et al., 2010a); single cell gel electrophoresis (SCGE) assay (Singh et al., 1988; Saquib et al., 2009); Cytokinesis blocked micronucleus (CBMN) assay (Kalantzi et al., 2004; Saquib et al., 2009) and flow cytometry (Saquib et al., 2010a, 2012) have been exploited. This study has elucidated some important and relatively unattended issues of toxicological significance, such as the (i) nature of butachlor–DNA interaction, (ii) extent of DNA strand breaks, (iii) induced ROS production and cytotoxicity and (iv) impact on cell cycle progression, eventually leading to cell apoptosis and/or necrosis.

2. Materials and methods

2.1. Chemicals

Butachlor [N-(butoxymethyl)-2-chloro-2′,6′-diethyl acetanilide] CAS No. 23184-66-9, 95% TC (Fig. 1) was a kind gift from the Agrochemical Division, (IARI, New Delhi, India). Deoxyribonucleic acid (DNA), sodium salt, highly polymerized (Type I) from calf thymus, low and normal melting temperature agarose (LMA and NMA), Na_2-EDTA, Tris-buffer, ethidium bromide (EtBr), propiodium iodide, methyl methane sulphonate (MMS), histopaque 1077, cytochalesin B (Cyto B), phytohemagglutinin-M (PHA-M), 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and DMSO were obtained from Sigma Chemical Company (St. Louis, MO, USA). DMSO (1%) was used as solvent control in experiments where specified, unless otherwise stated. RPMI-1640, foetal bovine serum (FBS) was procured from GIBCO BRL Life Technologies Inc. (Gaithersburg, MD, USA). Phosphate buffered saline (PBS, Ca^{2+} Mg^{2+} free) and Triton X-100 were obtained from Hi-Media Pvt. Ltd. (India). All other chemicals were of analytical grade. The slides for microgel electrophoresis were purchased from Blue Label Scientifics Pvt. Ltd., (Mumbai, India).

2.2. Butachlor–DNA binding analysis by fluorescence spectroscopy

Binding of butachlor to DNA was determined by use of fluorescence spectroscopy. Briefly, to a fixed concentration of butachlor (50 μM), increasing concentrations of ctDNA (5–100 μM) were added to obtain ctDNA to butachlor molar ratios ranging from 0.1 to 2.0 in 10 mM Tris–HCl buffer at ambient temperature. Spectra were recorded under subdued light to prevent undesired photodegradation. Fluorescence was determined by use of a Shimadzu spectrofluorophotometer, model RF5301PC equipped with RF 530XPC instrument control software, Kyoto (Japan). The path length was 1 cm in a quartz cell. Excitation and emission slits were set at 3 and 10 nm, respectively. The excitation and emission wavelengths were 225 and 360 nm, respectively. ctDNA alone does not fluoresce at this wavelength. The fluorescence quenching constant was determined by use of the Stern–Volmer relationship (Eq. (1)), as described previously (Lakowicz, 2006).

$$\frac{F_0}{F} = 1 + \mathrm{Ksv}\,[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher (ctDNA), respectively, Ksv is the Stern–Volmer quenching constant and [Q] the quencher concentration. The quenching constant was obtained from the slope of the Stern–Volmer plot (F_0/F vs [Q]). The binding constant (Ka) and number of binding sites (n) were estimated, following previously published methods (Lehrer and Fasman, 1996; Chipman et al., 1967) (Eq. (2)) and assuming a 1:1 complex between butachlor and ctDNA, as described previously (Saquib et al., 2010a, 2011).

$$\frac{F_0 - F}{F - F\infty} = \mathrm{Ka} \times [\mathrm{DNA}] \quad (2)$$

where F_0 and $F\infty$ are the relative fluorescence intensities of butachlor alone and butachlor saturated with ctDNA, expressed as the relative fluorescence intensity of ctDNA to butachlor molar ratio of 1:2, respectively. The slope of the linear portion of the double-logarithm plot ($\mathrm{Log}[(F_0 - F)/(F - F\infty)]$ vs Log [ctDNA] provided the number of equivalent binding sites (n). However, the value of Log [ctDNA] at $\mathrm{Log}[(F_0 - F)/(F - F\infty)] = 0$ is equal to the negative logarithm of the binding constant (Ka) (Lakowicz, 2006).

2.3. Measurement of DNA strand breaks in human PBMN cells by Comet assay

Comet assay was performed with human PBMN cells following the methods of Singh et al. (1988) as described by Saquib et al. (2009). Freshly isolated cells were treated separately with varying concentrations (50, 100, 250 and 500 μM) of butachlor for 3 h at 37 °C. The cells (~4×10^4) both untreated and treated were suspended in 100 μl of Ca^{2+} Mg^{2+} free PBS and mixed with 100 μl of 1% LMA. The cell suspension (80 μl) was then layered on one third frosted slides, pre-coated with NMA (1% in PBS without Ca^{2+} and Mg^{2+}) and kept at 4 °C for 10 min. After gelling, a layer of 90 μl of LMA (0.5% in PBS) was added. The cells were lysed in a lysing solution for overnight. After washing with Milli-Q water, the slides were subjected to DNA denaturation in cold electrophoresis buffer at 4 °C for 20 min. Electrophoresis was performed at 0.7 V/cm for 30 min (300 mA, 24 V) at 4 °C. The slides were then washed three times with neutralization buffer. All preparative steps were conducted in dark to prevent secondary DNA damage. The slides were stained with ethidium bromide for 5 min and analyzed at 40× magnification using fluorescence microscope (Olympus, Japan) coupled with charge coupled device (CCD) camera. Images from 50 cells (25 from each replicate slide) were randomly selected and subjected to image analysis using software Komet 3.0 (Kinetic Imaging, Liverpool, UK). The data were subjected to one-way analysis of variance (ANOVA). Mean values of the tail length (μm), Olive tail moment (OTM) and % tail DNA (% TDNA) were separately analyzed for statistical significance, level of statistical significance chosen was $p \leq 0.05$, unless otherwise stated.

2.4. Butachlor induced 8-oxo-2′-deoxyguanosine (8-oxodG) formation in ctDNA

Varying amounts (500, 1000, 1500 and 2000 ng) of untreated, butachlor (1000 μM) and methylene blue (100 μM) treated ctDNA samples were immobilized on 96 well microtiter plates by overnight absorption at 40 °C following the procedure of Hirayama et al. (1996). Methylene blue was used as positive control. The non-specific sites were blocked with 300 μl of blocking solution containing 3% BSA in PBS (Ca^{++} and Mg^{++} free). The plates were further incubated at 37 °C for 3 h. Polyclonal goat anti 8-oxodG antibodies (Cat # AHP592; AbDSerotech, UK), 100 μl/well at dilutions of 1:1,00,000 in blocking solution were added and incubated at 37 °C for 2 h. The solution was discarded and the plates washed twice with (300 μl/well) with PBS containing 0.05%Tween-20. Subsequently, the secondary antibody (Donkey anti-goat IgG:HRP; Cat # STAR88P, AbDSerotech, UK) diluted at 1:25,000 in