Differential Inhibition of UV-Induced Activation of NFκB and AP-1 by Extracts From Black Raspberries, Strawberries, and Blueberries

Chuanshu Huang, Dongyun Zhang, Jingxia Li, Qiangsong Tong, and Gary D. Stoner

Abstract: Recent studies from our laboratory have shown that the transactivation of nuclear factor κB (NFκB) and activator protein-1 (AP-1) plays an important mechanistic role in ultraviolet (UV)-induced skin carcinogenesis in mice. We also demonstrated that a methanol extract (ME) fraction from black raspberries (Rubus occidentalis) (RO; RO-ME) inhibits benzo[a]pyrene-7,8-diol-9,10-epoxide [B(a)PDE]–induced activation of NFκB and AP-1 in cultured mouse epidermal cells. In the present study, we determined if RO-ME might also inhibit the induction of NFκB and AP-1 in mouse epidermal cells exposed to mid UV radiation (UVB) and short UV radiation (UVC) and whether methanol fractions from strawberries and blueberries would also be effective. Our results showed that RO-ME inhibited UVB-induced activation of NFκB in mouse epidermal cells in a time- and dose-dependent manner; however, the methanol fractions from strawberries and blueberries were ineffective. Interestingly, none of the fractions from all 3 berry types inhibited UVB- or UVC-induced activation of AP-1, suggesting that inhibition of UV-induced signaling pathways is specific for black raspberries and NFκB. Cyanidin-3-rutinoside, an anthocyanin found in abundance in black raspberries and not in strawberries or high-bush blueberries, was found to contribute to the inhibition of UVB-induced activation of NFκB. These results suggest that berries differ in their ability to influence signaling pathways leading to activation of NFκB and AP-1 when using UV light as the inducer.

Introduction

Epidemiologic studies have revealed that ultraviolet (UV) radiation from the sun is the major cause of skin cancer and accounts for 1.3 million new cases of skin cancer each year in the United States (1). Reports from a number of laboratories demonstrated that UV radiation is a complete carcinogen with tumor-initiating as well as tumor-promoting potential (2). The exposure of mammalian cells to UV radiation, including short (UVC, 200-280 nm), long (UVA, 320–400 nm) and mid (UVB, 280-320 nm) wavelengths, leads to numerous changes in cells including damage to cell membranes (3), DNA damage (4), gene mutations (5), production of reactive oxygen species (6,7) and activation of kinases and their target transcription factors (8–11). These effects are generating considerable attention because of an alarming increase in the incidence of sunlight-related skin cancers (12). Although exposure to UV radiation can be minimized, it is highly unlikely that it will ever be completely eliminated. Thus, more effort should be directed toward identifying novel anticarcinogenic agents for preventing UV-induced skin cancer.

Chemoprevention by naturally occurring agents is gaining momentum in the management of neoplasia including skin cancer (13). A large body of evidence from case-control and cohort studies indicates that fruits and vegetables protect against various types of cancers (14,15). Blackberries, strawberries, and blueberries are well recognized for their potential health benefits (16). Dietary freeze-dried strawberries and black raspberries reduced the multiplicity of esophageal tumors in rats treated with the nitrosamine carcinogen, N-nitrosomethylbenzylamine(17–19). Furthermore, the observed reductions in O6-methylguanine adduct levels in esophageal DNA from rats fed with strawberries or black raspberries suggest that components in these berries influence the metabolism and DNA damaging effects of N-nitrosomethylbenzylamine (17,18). Many of the health-promoting properties of berries are thought to be attributable to their content of bioactive compounds such as the proanthocyanidins, anthocyanins, and ellagitannins (20). Dietary consumption of anthocyanins was shown to improve the overall antioxidant defense status of human plasma (21). Strawberries, black raspberries, and blueberries are high in antioxidant activity, and thus, their consumption has been shown to increase the antioxidant capacity of humans (22–24).

Our previous studies have shown that a methanol (ME) extract fraction from black raspberries [Rubus occidentalis] (RO-ME) inhibits benzo[a]pyrene (B[a]P)-induced transformation of Syrian hamster embryo cells (25) and...
benzo[a]pyrene-7,8-diol-9,10-epoxide (B[a]PDE)-induced transactivation of nuclear factor kappa B (NFκB) and activator protein-1 (AP-1) in JB6 mouse epidermal cells (26). More recently, we showed that extract fractions of black raspberries can block B[a]PDE-induced expression of VEGF in JB6 cells by impairing the phosphotidylinositol 3-kinase/Akt/AP-1 pathway (27). Since UV radiation induces transactivation of both NFκB and AP-1 (11,28,29), 2 genes of importance in skin tumor promotion and progression, we evaluated the potential effects of black raspberry, strawberry and blueberry extract fractions on UV-induced signaling pathways leading to the activation of NFκB and AP-1 in mouse epidermal cells.

Materials and Methods

Cell Culture, Reagents and Sources of UV Radiation

The cells used in this study were the mouse epidermal cell line JB6 Clone 41 (Cl 41) and 2 cell lines derived from Cl 41 cells and transfected stably with either a NFκB-luciferase reporter gene (Cl 41 NFκB mass 1 cells) or an AP-1-luciferase reporter gene (P+1-1 cells). All cells were cultured as monolayers in Eagle’s Minimal Essential Medium (MEM; Calbiochem, San Diego, CA) containing 5% fetal bovine serum (FBS; Life Technologies, Inc., Carlsbad, CA), 2 mM L-glutamine, and 25 μg gentamicin/ml (26). Once or twice per week, the cultures were dissociated with a 0.25% trypsin solution and transferred to new 75-cm² culture flasks (Fisher, Pittsburgh, PA). Cyanidin-3-rutinoside (CyR) was purchased from Extrasynthese (Genay, France) and luciferase assay substrate was purchased from Promega (Madison, WI). UV radiation lamps were purchased from UVP, Inc. (Upland, CA). UVC lamps generate UV light at 254 nm wavelength, whereas UVB lamps generate >95% of UVB light at 302 nm wavelength and some UVC light. The UVB radiation used in this study was filtered with a Kodak Kodacel K6808 filter (Eastman Kodak, Rochester, NY) that eliminates all wavelengths <290 nm (28,30).

Berries and Fractions

Fractions of black raspberries, strawberries, and blueberries were prepared as previously described (25,26). Briefly, ripe black raspberries (Rubus occidentalis; RO), strawberries (Fragaria ananassa; FA), and blueberries (Vaccinium corymbosum; VC) were washed immediately after picking and frozen at −20°C. Approximately 1 lb of freeze-dried berries of each type was extracted in 3 volumes of methanol overnight for 3 nights. The extract was filtered and then dried under vacuum at 60°C to produce fraction F001. A portion of F001 was partitioned with water:dichloromethane (1:1). The resulting nonpolar elute (DM) and polar fraction (ME) were obtained. All extracts were stored at −20°C in the dark. For cell treatment, each extract was dissolved in dimethyl sulfoxide to a stock concentration of 50 mg/ml and frozen at −70°C.

NFκB and AP-1 Activity Assay

Confluent monolayers of Cl 41 cells transfected with NFκB-luciferase reporter (NFκB mass1) or Cl 41 cells transfected with AP-1-luciferase reporter (P+1-1) (26) were treated with trypsin, and 8 × 10⁵ viable cells suspended in 100 μl medium were added to each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO₂. The cells were pretreated with berry fractions for 30 min and then exposed to UV radiation for NFκB or AP-1 induction. After different times, cells were extracted with 50 μl lysis buffer for 30 min at 4°C, and luciferase activity was measured using Promega luciferase assay reagent with a luminometer (Wallace 1420 Victor 2 multiplicable counter system, Perkin Elmer Life Science, Boston, MA). Results are expressed as NFκB or AP-1 activity relative to control medium (relative NFκB or AP-1 activity) (26).

Kinase Phosphorylation Assay

Cl 41 cells (5 × 10⁵) were cultured in each well of 6-well plates to 70–80% confluence with 5% FBS MEM. The medium was replaced with MEM supplemented with 0.1% FBS and cultured for 45 h. Cells were then incubated in 0.1% FBS MEM for 3–4 h at 37°C. Cells were pretreated with berry extract fractions for 30 min, exposed to 2KJ/m² UVB for 30–180 min, washed once with ice-cold phosphate buffered saline, and extracted with a sodium dodecyl sulfate (SDS)-sample buffer. Western blots were performed with either phospho-specific or nonphosphorylated antibodies against various proteins including IκB kinase beta (IKKβ), c-Jun, extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 kinase (p38K). The protein band specifically bound to the primary antibody was detected with an antirabbit immunoglobulin-AP-linked secondary antibody and enhanced chemiluminescence (ECF) Western blotting system (Amersham Biosciences, Piscataway, NJ).

Statistical Analysis

The Student t-test with a Bonferroni correction was used to determine the significance of the differences for NFκB and AP-1 activities in cells treated with the berry fractions.

Results

Effects of Fractions From Black Raspberries, Strawberries and Blueberries on UV-Induced NFκB Transactivation

Pretreatment of Cl 41 NFκB mass1 cells with either the methanol-soluble (RO-ME) or the water-soluble...
Figure 1. Inhibition of nuclear factor kappaB (NFκB) activation by extracts from black raspberries but not strawberries and blueberries. A-E: Cl 41 NFκB mass1 cells were seeded into each well of 96-well plates and cultured in Eagle’s minimal essential medium containing 5% fetal bovine serum at 37°C. After the cell density reached 80–90%, the cells were first pretreated for 30 min with various berry extract fractions. The cultures were then exposed to mid ultraviolet (UV) radiation (UVB) (2 KJ/m²) for NFκB induction. At 36 h after UV exposure, the cells were extracted with 50 µl lysis buffer for 30 min at 4°C for luciferase activity measure. The luciferase assay results are expressed as NFκB activity relative to medium control. Each bar indicates the mean and SE of triplicate assay wells. The asterisk (*) indicates a significant decrease from UV exposure. The symbol ♣ indicates a significant increase from UV exposure. F: Cl 41 cells were seeded into each well of 6-well plates. After the cell density reached 80–90%, the cells were first pretreated for 30 min with various berry extract fractions and then exposed to UVB (2 KJ/m²). At 30 min later, the cells were extracted with sodium dodecyl sulfate sample and subjected to Western blotting analysis. AP-1, activator protein-1; RO-ME, Rubus occidentalis (RO) methanol extract (ME) fraction from black raspberries; UVC, short UV radiation; FA, Fagra ananassa; VA, Vaccinium corymbosu.

(RO-FOO3) fractions of black raspberries resulted in a marked inhibition of UVB-induced activation of NFκB in a time-dependent manner (Figs. 1A and 1B). In contrast, another black raspberry fraction RO-DM actually enhanced NFκB activation. Interestingly, the methanol fractions from strawberries (FA-ME) and blueberries (VC-ME) failed to inhibit UVB-induced NFκB activation (Figs. 1C and 1D), and the water-soluble fractions from these same berry types were also ineffective (data not shown). Dose-response studies confirmed that RO-ME was the most potent inhibitor of UVB-induced NFκB activation amongst the methanol fractions of the three berry types (Fig. 1E). The RO-ME fraction was also the most effective inhibitor of UVC-induced NFκB activation in Cl 41 cells among the methanol fractions of the 3 berry types (data not shown). Potent NFκB activators such as UVB exposure resulted in activation of the IKK, which phosphorylates IκB leading to degradation of IκB and activation of NFκB (31,32). As shown in Fig. 1F, exposure to 2KJ/m² UVB results in obvious phosphorylation of IKKβ at Ser-181, which indicates IKK activation. Pretreatment with black raspberry fraction RO-ME significantly reduced IKKβ phosphorylation. In contrast, methanol fractions from strawberries (FA-ME) or blueberries (VC-ME) only showed marginal effect on IKKβ phosphorylation. These data demonstrate that black raspberry fraction RO-ME blocks UVB exposure-induced NFκB transactivation through...
Figure 2. No inhibition of activator protein-1 (AP-1) activation by extracts from black raspberries, strawberries, or blueberries. P1-1 cells (8 × 10^3) were seeded into each well of 96-well plates and cultured in Eagle’s minimal essential medium containing 5% fetal bovine serum at 37°C. After the cell density reached 80–90%, the cells were first pretreated for 30 min with 50 µg/ml of various extract fractions from black raspberries (A and B), strawberries (C and D), different concentrations of methanol extract (ME) fractions from black raspberries Rubus occidentalis (RO; RO-ME), Fragara ananassa strawberries (FA; FA-ME), or blueberries Vaccinium corymbosu (VC; VC-ME), or treated with RO-ME (50 µg/ml) at 30 min before ultraviolet (UV) exposure simultaneously with UV radiation or 3 h after UV exposure (F). The cultures were then exposed to mid UV radiation (UVB) (2 KJ/m²) or short UV radiation (UVC) (30 J/m²) for AP-1 induction. At 24 h after UV exposure, the cells were extracted with 50 µl lysis buffer for 30 min at 4°C, and luciferase activity was measured. The results are expressed as AP-1 activity relative to medium control. Each bar indicates the mean and SE of triplicate assay wells. The symbol ♣ indicates a significant increase from UV exposure. DM, dichloromethan.

suppression of IKK activation. In contrast, strawberry extract FA-ME and blueberry extract VC-ME failed to repress UVB-induced activation of the IKK/NFκB pathway.

Effects of Fractions From Black Raspberries, Strawberries and Blueberries on UV-Induced AP-1 Transactivation

Our previous studies have shown that RO-ME inhibits B[a]PDE-induced AP-1 and NFκB activation when RO-ME is preincubated for 30 min or added simultaneously with B[a]PDE (26). However, none of the black raspberry fractions, including RO-ME, inhibited UVB-induced AP-1 activation (Fig. 2A). In fact, fractions RO-FOO3, RO-FOO4, and RO-DM actually enhanced AP-1 activation. In addition, none of the strawberry fractions inhibited UVB-induced AP-1 activation (Fig. 2C), and fractions FA-F003, FA-FOO4 and FA-DM enhanced AP-1 activation induced by UVB radiation. Similar results were observed in effects of those fractions on UVC-induced AP-1 activation (Figs. 2B and 2D), suggesting that the failure of the inhibition by black raspberry extract fractions on UV-induced AP-1 activation is not UV wavelength dependent. The results obtained from the corresponding fractions of blueberries also did not show any inhibitory effect on UV-induced AP-1 activity (data not shown). Furthermore, data from dose-response studies and from treatment of cells with berry fractions either before,
Figure 3. Incubation of cells with methanol extracts (ME) from black raspberries Rubus occidentalis (RO; RO-ME), strawberries Fragaria ananassa ME (FA; FA-ME) and blueberries Vaccinium corymbosu (VC; VC-ME) did not inhibit ultraviolet (UV)-induced MAPK/c-Jun pathway activation. Cl41 cells were seeded into each well of 6-well plates and cultured in Eagle’s minimal essential medium (MEM) containing 5% fetal bovine serum at 37°C until cell density reached 70–80%. The cell culture medium was replaced with 0.1% FBS MEM. At 45 h later, cells were incubated with fresh serum-free MEM for 3–4 h at 37°C. Cells were pretreated with indicated berry fractions at 50 µg/ml for 30 min, exposed to mid UV radiation (UVB) (2 KJ/m²) for 30–180 min, and extracted with an sodium dodecyl sulfate sample buffer and Western blot analysis carried out as described in Materials and Methods. JNKs, c-Jun N-terminal kinases; ERKs, extracellular signal-regulated kinases.

simultaneously, or after UV exposure have also confirmed these findings (Figs. 2E and 2F). Because c-Jun is the main component of the AP-1 complex, we tested the effect of berry extracts on c-Jun activation. As shown in Fig. 3A, UVB exposure led to significant c-Jun phosphorylation at Ser-63 and Ser-73; however, pretreatment with each of the berry extracts failed to affect c-Jun phosphorylation. These results are consistent with the lack of berry effects on AP-1 transactivation. Mitogen-activated protein kinases (MAPK) are responsible for c-Jun activation, and pretreatment with methanol fractions from all 3 berry types did not lead to suppression of UVB-induced activation of JNKs, p38K, and ERKs (Fig. 3B). The failure of all 3 berry fractions to inhibit UV-induced AP-1 activation suggests that the observed inhibition of UV-induced NFκB activation by RO-ME is specific to black raspberries and to NFκB.

**Effect of CyR on UV-Induced Transactivation of NFκB in Mouse Cl 41 Cells**

Anthocyanins are one of the most abundant phenolics in nature and are responsible for the red, purple, and blue colors of most fruits and vegetables (33). Consumption of anthocyanin-rich foods may contribute to the protective effects of these foods against degenerative conditions such as cardiovascular disease and cancer (34). Our collaborators determined that CyR is the most abundant anthocyanin in freeze-dried black raspberries (35). CyR is also the most abundant anthocyanin in alcohol extracts of black raspberries (unpublished data). In both freeze-dried black raspberries and in the methanol fraction, CyR represents about 60% of the total anthocyanins, and cyanidin-3-glucoside, cyanidin-2β-xylosylrutinoside, and cyanidin-3-sambubioside represent about 16%, 20%, and 4%, respectively, of the total anthocyanins. Thus, the relative proportions of the anthocyanins in the methanol fraction closely parallel those in the freeze-dried berries themselves. Given this information, we determined whether pretreatment with CyR would result in inhibition of UVB-induced NFκB activation in JB6 Cl 41 cells. Pretreatment of Cl 41 cells with a noncytotoxic concentration of CyR resulted in a dramatic decrease in UVB-induced NFκB activation (Fig. 4A), and this decrease was dose dependent (Fig. 4B). CyR treatment alone did not affect the basal level of NFκB activity (Fig. 4B). These results suggest that at least one anthocyanin in black raspberries inhibits NFκB activation induced by UVB in Cl 41 cells.

**Discussion**

Considerable research is underway to identify the anticarcinogenic compounds and phytochemicals responsible for the cancer-preventing action of fruits and vegetables. Berry fruits have shown a remarkably high scavenging activity for chemically generated radicals (36–38), and edible berries exhibit potent chemopreventive activities (39). Previous studies have shown that a methanol fraction (RO-ME) from freeze-dried black raspberries inhibits B[a]P-induced cell transformation (25) and B[a]PDE-induced transactivation of AP-1 and NFκB in mouse epidermal Cl 41 cells (26). There are no reports, however, of whether extract fractions from other different berry types can inhibit UV-induced signaling pathways. In this study, we found that black raspberry fraction RO-ME specifically inhibits transactivation of NFκB but does not inhibit UV-induced activation of AP-1, which plays an important role in UV-induced carcinogenesis (40). In contrast, methanol fractions, FA-ME and VC-ME, from strawberries and blueberries, respectively, did not inhibit UV-induced activation of NFκB or AP-1.
Figure 4. Inhibition of nuclear factor kappaB (NFκB) activation by cyanidin-3-rutinoside (CyR) in mouse Cl 41 cells. Cl 41 NFκB mass1 cells were seeded into each well of 96-well plates and cultured in Eagle’s minimal essential medium containing 5% fetal bovine serum at 37°C. After the cell density reached 80–90%, the cells were first pretreated with 32 µg/ml (A) or various concentrations (B) of CyR for 30 min and then exposed to mid ultraviolet (UV) radiation (UVB) (2 KJ/m²) for NFκB induction. At 36 h after UV exposure, the cells were extracted with 50 µl lysis buffer for 30 min at 4°C, and luciferase activity was measured. The results are expressed as NFκB activity relative to medium control. Each bar indicates the mean and SE of triplicate assay wells. The asterisk (*) indicates a significant decrease from UV radiation.

RO-ME is capable of inhibiting NFκB transactivation induced by B[a]PDE (26) as well as by UV (present study). The inhibitory effect of RO-ME appears to be due to the suppression of the phosphorylation of IKKβ (Fig. 1F), a molecule that is involved in both B[a]PDE- and UVB-induced activation of NFκB (26, 41). In contrast, methanol fractions FA-ME and VC-ME from strawberries and blueberries, respectively, are unable to interfere with UVB-induced NFκB transactivation (Figs. 1C–1E). These findings are consistent with their inability to suppress IKKβ phosphorylation (Fig. 1F). Recently, we have also found that neither FA-ME nor VC-ME inhibit B[a]PDE-induced transactivation of NFκB (unpublished data).

Interestingly, although RO-ME shows significant inhibitory effect on B[a]PDE-induced AP-1 transactivation (26), it fails to affect UVB/UVC-induced AP-1 activation (Figs. 2A and 2B). The differential signaling pathways involved in AP-1 activation by the 2 inducers may underlie this discrepancy. Our recent studies reveal that B[a]PDE-induced AP-1 activation is through cascades activation of the PI-3K/Akt/JNK pathway, and RO-ME blocks the initiation step of this pathway at PI-3K level (28, 42). This would lead to the observed blockage of AP-1 and VEGF, which are downstream of PI-3K (26). In contrast, we report that UVB-induced AP-1 activation in Cl 41 cells requires activation of ERKs (10). Interestingly, RO-ME, FA-ME, and VC-ME are all unable to inhibit UVB-induced ERKs phosphorylation (Fig. 3B), which may explain their inability to inhibit AP-1 activation. These fractions also fail to inhibit UVB-induced activation of p38K and JNKs (Fig. 3B), which is consistent with their lack of effect on c-Jun activation (Fig. 3A).

As mentioned, in the present study, RO-ME targeted only UV-induced NFκB activation and had no effect on AP-1 activation. Similar to these results, Pendruth et al. (43) report that resveratrol, a polyphenol in red wine with chemoprevention properties, suppresses LPS-induced transactivation of NFκB leading to suppression of downstream genes such as TNF-α. Resveratrol, however, has no effect on AP-1-mediated transcriptional activity. It seems plausible, therefore, that NFκB and AP-1 transactivations require different signaling pathways, and RO-ME and resveratrol interfere only with NFκB pathways and not AP-1 pathways.

Edible berry anthocyanins possess a broad spectrum of therapeutic properties such as antioxidant, antiinflammatory, anticarcinogenic, and neuroprotective actions (44). Cyanidin is shown recently to inhibit the growth of human vulva carcinoma cells, in part by inhibiting the epidermal growth factor receptor through shutting off downstream signaling cascades (45). Recently, Stoner et al. (46) determined that there are 4 major anthocyanins in black raspberries, and CyR is the most abundant cyanidin (see also Ref. 35). In this study, CyR, representing approximately 60% of the anthocyanins in RO-ME, inhibits UVB-induced activation of NFκB (Fig. 4). These data suggest that CyR and possibly other anthocyanins in black raspberries might contribute to the inhibition of UV-induced NFκB activation by RO-ME. Ripe black raspberries have higher anthocyanin and total phenolic contents than either strawberries or blueberries (47–50). Moreover, they have a different profile of anthocyanins than are found in strawberries and blueberries (46,51,52). Thus, we hypothesize that the differential inhibitory effects of black raspberries, strawberries, and blueberries on UV-induced activation of NFκB may be related to differences in their contents and profiles of anthocyanins such as CyR. Here we need to mention that some polyacylated anthocyanins with dark color can screen UVB then reduce the harm of UVB.
efficiently due to aromatic acyl residues (53), while some other anthocyanins, such as cyanidin-3-O-glucoside, protect UVB irradiation-induced cell damages by the modulation of NFκB and AP-1 activation and the expression of the downstream target genes such as proinflammatory cytokine IL-8 (54). Our results showed that RO-ME specifically inhibited UV-induced NFκB activation, whereas it did not show any inhibition of UV-induced AP-1 activity. Those results strongly suggest that RO-ME reduced NFκB activation through the intracellular events such as signaling transduction pathways rather than physical filtering. This notion is also supported by our previous published findings that RO-ME can also suppress B[a]PDE-induced activation of NFκB and AP-1 (26).

In summary, the present study demonstrates that the RO-ME fraction from black raspberries appears to be responsible for inhibition of UV-induced activation of NFκB but not AP-1 in Cl 41 cells. Methanol fractions from strawberries and blueberries are ineffective. The differences among the berry types in their ability to inhibit UV-induced activation of NFκB may be due to their content of anthocyanins. Interestingly, none of the extract fractions from all 3 berry types inhibits UV-induced AP-1 activation in Cl 41 cells, suggesting that they do not influence signaling pathways involved in AP-1 activation. The molecular basis for these results is under investigation.

Acknowledgments and Notes

This work was supported in part by NIH/NIH Grants CA103180, CA094964, and CA112557 and NIH/NIH Grant ES012451. Address correspondence to Dr. Chuanshu Huang, Nelson Institute of Environmental Medicine, New York University School of Medicine, 57 Old Forge Road, Tuxedo, NY 10987. Phone: 845-731-3519. FAX: 845-351-2320. E-mail: chuanshu@env.med.nyu.edu.

Submitted 11 September 2006; accepted in final form 5 February 2007.

References


