Valproic acid, a histone deacetylase inhibitor, enhances sensitivity to doxorubicin in anaplastic thyroid cancer cells

Maria G Catalano1, Nicoletta Fortunati2, Mariateresa Pugliese2, Roberta Poli2, Ornella Bosco1, Raffaella Mastrocola3, Manuela Aragno3 and Giuseppe Boccuzzi1,2

1Department of Clinical Pathophysiology, University of Turin, Via Genova 3, 10126 Turin, Italy
2Oncological Endocrinology, ASO San Giovanni Battista via Genova 3, 10126 Turin, Italy
3Department of Experimental Medicine and Oncology, University of Turin, C.so Rafaele 30, 10125 Turin, Italy

Abstract

Multimodality treatments (i.e. surgery, chemotherapy, and radiotherapy) are recommended for anaplastic thyroid carcinoma (ATC), an extremely lethal human cancer, but to date there is little evidence that such approaches improve survival rates. It is thus necessary to seek new therapeutic tools. Histone deacetylase (HDAC) inhibitors are a promising class of anti-neoplastic agents that induce differentiation and apoptosis. Moreover, they may enhance the cytotoxicity of drugs targeting DNA through acetylation of histones. Using two ATC cell lines (CAL-62 and ARO), we show here that valproic acid (VPA), a clinically available HDAC inhibitor, enhances the activity of doxorubicin, whose anti-tumor properties involve binding to DNA and inhibiting topoisomerase II. A meager 0.7 mM VPA, which corresponds to serum concentrations in patients treated for epilepsy, is able to increase the cytotoxicity of doxorubicin about threefold in CAL-62 cells and twofold in ARO cells. The sensitizing effect, which is through histone acetylation, involves increased apoptosis, which is also shown by the increased caspase 3 activation and the enhancement of doxorubicin-induced G2 cell cycle arrest. These results might offer a rationale for clinical studies of a new combined therapy in an effort to improve the outcome of patients with anaplastic thyroid cancer.


Introduction

Anaplastic thyroid carcinoma (ATC) is one of the most aggressive human malignancies with a rapid and lethal clinical course (Pasiela 2003). ATC accounts for <5% of thyroid tumors but causes 14–39% of thyroid carcinoma deaths (Kitamura et al. 1999). Diagnosis of ATC is usually fatal, with a median survival of 3–9 months and only 10–15% of patients still alive after 2 years (Veness et al. 2004). The present American Joint Commission classifies all cases of ATC as stage IV (Fleming 1997). Treatment of patients with ATC is not standardized; multimodal therapy, including surgical resection, radiotherapy, and chemotherapy, is usually applied but it is unclear whether such an approach significantly improves survival (Busnardo et al. 2000, Haigh et al. 2001). As far as chemotherapy is concerned doxorubicin is the most frequently used drug, but monotherapy has a response rate below 20% and combination regimes have also unfortunately provided very little improvement in clinical response (Haigh 2000). Thus far, none of the drugs used seems to alter the fatal outcome of the disease; hence, new therapeutic approaches are needed.

Histone deacetylase (HDAC) inhibitors have emerged as a promising new class of anti-neoplastic agents (Marks et al. 2001a,b, Vigushin & Coombes 2002, Rosato & Grant 2003), and both natural and synthetic inhibitors have been characterized (De Ruiter et al. 2003). They share the capacity to promote differentiation, cell-cycle arrest and apoptosis (Lindemann et al. 2004). Moreover, HDAC inhibitors, through the hyperacetylation of histones and subsequent relaxation of chromatin, may enhance the cytotoxicity of drugs targeting DNA (Kim et al. 2003, Marchion et al. 2004, 2005), such as doxorubicin, whose anti-tumor activities involve binding to DNA and inhibiting topoisomerase II (Tewey et al. 1984). However, a number of limitations hamper the clinical use of HDAC inhibitors; some have a short half-life and/or significant toxic side effects in vivo (Marks et al. 2001a,b). Conversely, valproic acid (VPA), a potent anti-convulsant widely used to treat epilepsy and mood disorders that acts as an HDAC inhibitor at therapeutic concentrations (Gottlicher et al. 2001, Phil et al. 2001), produces mild adverse effects in man even when serum levels exceed the normal therapeutic range. We recently showed that, at concentrations reached in the serum of patients treated for epilepsy, VPA promotes iodine uptake (Fortunati et al. 2004) and controls cell growth (Catalano et al. 2005) in vitro in poorly differentiated thyroid cancer cells. In addition to its redifferentiating property, VPA has been reported to affect the growth...
of several transformed cells (Slesinger & Singer 1987, Knupfer et al. 1988, Abdul & Hoosen 2001, Ohen et al. 2004), and to induce apoptosis in human leukemia cell lines (Kawagoe et al. 2002) and endometrial cancer cells (Takai et al. 2004).

In the present study, we investigated the anti-neoplastic activity of VPA in anaplastic thyroid cancer cell lines and its capacity to increase the cytotoxicity of doxorubicin, a drug targeting DNA. Data indicate that VPA, which alone is ineffective against ATC cells, notably enhances doxorubicin-dependent apoptosis and cell cycle arrest.

Materials and Methods

Cell lines and culture conditions

ATC (CAL-62) cell line was purchased from Deutsche Sammlung von Mikroorganismen and Zellculturen (Braunschweig, Germany). ATC (ARO) cells were a kind gift from Prof. Mauro Papotti and Dr Paola Cassoni (Pathology Service, Department of Oncology, University of Turin). CAL-62 cells were routinely maintained in 25 cm² flasks at 37 °C, in 5% CO₂ and 95% humidity, with 100 IU/ml penicillin and 100 µg/ml streptomycin added, in DMEM-F12 (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Euroclone, Wetherby, West Yorks, UK). ARO cell line was maintained in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated FCS.

Western-blot analysis of histone acetylation

ARO and CAL-62 cells were evaluated for acetylated histone H4 in the presence of VPA by western blot. Cells were treated with 0-7 mM VPA for 24 h and harvested. In 80 µl lysis buffer (50 mM Tris–HCl, pH 6-8, 10% glycerol, 4% β-mercaptoethanol, and 1% SDS), 5×10⁶ cells were lysed in the presence of protease inhibitors, sonicated, and centrifuged for 20 min at 12 000 g at 4 °C. An equal volume of 2× SDS gel loading buffer was added to 5 µl supernatant and the sample was boiled for 5 min. The proteins were separated on 15% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membrane and probed with the following primary antibodies: rabbit polyclonal anti-acetyl-histone H4 (06-598, 1:1000 dilution, Upstate, Lake Placid, NY, USA) and mouse monoclonal anti-actin (Monoclonal Anti-actin clone AC-40, 1:1000 dilution, Sigma) to check protein loading. Proteins were detected with Pierce Super Signal chemiluminescent substrate following the manufacturer’s instructions (Pierce, Rockford, IL, USA). Bands were photographed and analyzed using the Kodak 1D Image Analysis software.

Cell viability assay

To evaluate the effect of VPA on cell viability, CAL-62 and ARO cells were seeded at 3×10⁶ cells/well in 96-well plates (Corning, New York, NY, USA) in culture medium plus 10% FCS. After 24 h, the cells were treated with medium in the absence or the presence of VPA (0.5–1.5 mM) (Sigma) for up to 11 days. At days 1, 4, 7, 9, and 11, viable cells were determined using the Cell Proliferation Reagent WST-1 (Roche), following the manufacturer’s instructions. This is a colorimetric assay for the quantification of cell viability and proliferation, based on cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. Briefly, 10 µl WST-1 were added to each well. After 1-h incubation, absorbance at 450 nm was measured using a plate reader (Model 680 Microplate Reader, Bio–Rad). Four replicate wells were used to determine each data point.

To evaluate the effect of VPA on cytotoxicity induced by doxorubicin, cells were exposed to either 0.7 or 1.5 mM VPA for 24 h before the addition of doxorubicin (0–1 µM). After a further 72-h incubation, cell viability was assessed as described above.

Apoptosis detection

Cell death detection ELISA For apoptotic studies, 3×10⁵ cells were seeded in 96-well plates and treated with VPA and doxorubicin as for the viability assay; apoptosis was evaluated using Cell Death Detection ELISA (Roche) following the manufacturer's instructions. This assay is based on a quantitative sandwich–enzyme-immunoassay principle using monoclonal antibodies directed against DNA and histones respectively. The assay provides the specific determination of mono- and oligonucleosomes in the cytoplasm fraction of cell lysates. Apoptosis was expressed as enrichment factor, calculated as a fraction of the absorbance of treated cells versus untreated controls.

Caspase activity assay In 75 cm² flasks, 1×10⁶ cells were seeded and exposed to VPA and doxorubicin as above. After drug treatments, caspase 3 was determined using a colorimetric assay kit (R&D Systems Inc., Minneapolis, MN, USA) following the manufacturer's instructions. Briefly, cells were lysed and incubated with the colorimetric substrate DEVD-pNA for 2 h at 37 °C. After incubation, the chromophore was quantified spectrophotometrically at 405 nm.

Doxorubicin uptake

Both cell lines were exposed to 0-7 mM VPA for either 24 or 48 h followed by doxorubicin (0–1 µM) for either 6 or 24 h. After treatment, cells were harvested by trypsinization and washed thrice with PBS. Nuclei were purified as described elsewhere (Parker & Topol 1984). Nuclear fluorescence was determined using an EPICS XL flow cytometer (Coulter Corp. Hialeah, FL, USA).
Topoisomerase II gene expression

Cells were treated with increasing doses of VPA (0.5–1.5 mM) for up to 72 h. Total RNA was extracted at different times (24, 48, and 72 h) from both cell lines using TRIzol Reagent (Invitrogen) following the method developed by Chomczynski & Sacchi (1987). Total RNA was reverse-transcribed at 42 °C for 40 min using AMV reverse transcriptase (Finnzymes, Espoo, Finland) and oligo(dT) primer (Invitrogen). The PCR system contained 5 µl 10X PCR buffer, 10 µl RT product, 0.2 mM dNTP (Finnzymes), 1.25 U Taq DNA polymerase (Finnzymes). Primers: 5'-CGTCAGAACATGGACC and 3'-AGCAGATTCAGCACC for topoisomerase II α; 5'-CTCCTGCATGCTGGT and 3'-CTTGGCTGATCCATC for β-actin were used. Amplification was carried out as follows: 1× (95 °C, 1 min); 28× (95 °C, 30 s; 58 °C, 30 s; 72 °C, 30 s); and 1× (72 °C, 7 min). PCR products were electrophoresed on 1.5% agarose gel in the presence of ethidium bromide. Gels were photographed and analyzed with Kodak 1D Image Analysis software.

Western-blot analysis of topoisomerase II protein

In 75 cm² flasks, 1×10⁶ cells were seeded and treated with 0.5–1.5 mM VPA for 3 days. After treatment, nuclear protein extract was obtained as previously described (Parker & Topol 1984). Equal amounts of protein (50 µg protein/lane) were subjected to SDS-PAGE (T=6%) and electroblotted onto a PVDF membrane; the membrane was probed with the following primary antibodies: anti-human topoisomerase II (K-19, s.c.-5347, 1:400 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); anti-actin (Monoclonal Anti-actin clone AC-40, 1:1000 dilution, Sigma) to check protein loading.

Proteins were detected with enhanced chemo luminescence (ECL) western blot reagents following the manufacturer's instructions (Amersham Bioscience). Bands were photographed using the PC software 1D Kodak Digital Science.

Cell cycle analysis

Cells were treated with 0.7 mM VPA for 24 h, followed by treatment with 50 nM doxorubicin up to 72 h. After

Figure 1 VPA effect on cell viability and histone acetylation (A) viability of CAL-62 and ARO cells, after treatment with 0.5, 1, or 1.5 mM VPA for up to 11 days. Cell viability was determined as the ratio between treated cells and untreated controls (basal). Results are expressed as mean±S.E.M., n=3. (B) Accumulation of acetylated H4 (Ac-H4) histone in CAL-62 and ARO cells, incubated for 24 h with 0.7 mM VPA, was assessed by western blotting with an anti-acetyl-histone H4 antibody. Equal loading and transfer were verified by reprobing the membranes with an anti-actin antibody. Histogram: net intensity was determined as the ratio between VPA-treated cells and untreated control. The figure shows a typical experiment.
treatments, all cells were collected, fixed in 70% ethanol for 30 min on ice and incubated in propidium iodide solution (20 μg/ml propidium iodide, 0.2 mg/ml RNAseA in PBS) for 1 h at room temperature. The cell population was analyzed by flow cytometry.

**Oxidative state**

CAL-62 cells were treated with 0.7 mM VPA for 24 h, followed by doxorubicin and VPA for a further 24-h period. Reactive oxygen species (ROS) were then measured in culture media using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) probe. DCFH-DA is a stable, non-fluorescent molecule that is hydrolyzed by esterases to non-fluorescent 2′,7′-dichlorofluorescein (DCF), which is rapidly oxidized in the presence of peroxides to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). DCF is measured fluorimetrically (Ravindranath 1994) at 488 nm excitation and 525 nm emission.

**Statistical analysis**

Data are expressed throughout as means ± S.E.M., calculated from at least three different experiments. Statistical comparisons between groups were performed with one-way ANOVA and the threshold of significance was calculated with the Bonferroni test. Caspase activities were compared through the paired t-test. Statistical significance was set at $P<0.05$.

**Results**

**Effects of VPA on cell growth and acetylation of histones**

We first evaluated the effect of treatment with VPA alone on cell viability in both the anaplastic thyroid cancer cell lines. Cell growth of either CAL-62 or ARO cells was not affected after treatment with increasing doses of VPA up to 1.5 mM for 11 days (one-way ANOVA test, Fig. 1A). Despite having no effects on cell growth, treatment of either cell line with VPA resulted in acetylation of H4, as reported in Fig. 1B. This dose of VPA and this exposure time were thus chosen to study the ability of VPA, through chromatin relaxation, to increase the cytotoxicity of doxorubicin, a drug targeting DNA.

**Effect of VPA on doxorubicin-induced cytotoxicity**

The effect on cell viability of doxorubicin alone or after pretreatment with VPA is reported in Fig. 2. Pre-treatment of the cells with either 0.7 or 1.5 mM VPA significantly increased the effect of doxorubicin in both the cell lines. In CAL-62 cells, 0.5 μM doxorubicin alone left 48% of cells surviving compared with 15% after VPA pre-treatment. As far as ARO cells are concerned, 1 μM doxorubicin alone left 43% of cells surviving compared with 20% with VPA pre-treatment. No significant difference was observed between the doses of 0.7 and 1.5 mM VPA.

**Effect of VPA on doxorubicin-mediated apoptosis**

Since our data indicate that pre-treatment of CAL-62 and ARO cells with VPA sensitizes cells to doxorubicin, we investigated whether the combined treatment affected the induction of apoptosis. As shown in Fig. 3, upper panels, VPA alone had no effect on apoptosis, while it significantly enhanced doxorubicin-mediated apoptosis in both the cell lines and at all concentrations used. The induction of apoptosis was further confirmed by caspase 3 activation; as reported in Fig. 3, lower panels, VPA alone had no effect on caspase 3 activation, while doxorubicin significantly activated this caspase in both the cell lines ($P<0.001$). Pre-treatment with VPA caused a further increase in the caspase 3 activation induced by doxorubicin ($P<0.001$ in CAL-62 cells, $P<0.01$ in ARO cells). Results are expressed as means ± S.E.M.; $n = 3$.

**Effect of VPA on doxorubicin uptake and topoisomerase II**

VPA’s inhibition of deacetylation may increase doxorubicin accessibility to DNA; however, in our experimental conditions, VPA pre-treatment did not modify the amount of nuclear doxorubicin (data not shown).

It is also reported that doxorubicin cytotoxicity is increased by higher topoisomerase II levels (Kurz et al. 2001), since this drug...
is a topoisomerase II inhibitor which is able to convert the enzyme reversible interaction with DNA into covalent adducts that result in DNA damage. However, VPA treatment for up to 72 h did not determine any significant modification either in the expression of topoisomerase mRNA (Fig. 4) or in the protein levels (Fig. 5), thus it is unlikely that the effect of VPA takes place through increased enzyme levels.

Effect of VPA on doxorubicin-induced G2 cell cycle arrest

The effect of doxorubicin on cell growth is also mediated by a G2 cell cycle arrest, and we therefore assessed the effect of VPA pre-treatment on cell cycle progression. As shown in Table 1, VPA, already after 24-h treatment, significantly enhanced the G2 arrest induced by doxorubicin in CAL-62. A total of 7-9% of untreated CAL-62 cells were in G2 compared with 50 nM doxorubicin alone and 58% of those pre-treated with 0.7 mM VPA (VPA + doxorubicin, P<0.01). VPA alone had no effect on G2 arrest. In addition, after 72-h treatment, a significant increase in sub-G1 was observed after VPA pre-treatment, indicating that cells arrested in G2 progressed to apoptosis (VPA + doxorubicin versus doxorubicin, P<0.01). The same effects were observed in ARO cells (data not shown).

<table>
<thead>
<tr>
<th>VPA (mM) 24 h</th>
<th>VPA (mM) 48 h</th>
<th>VPA (mM) 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 0.5 1 1.5 1</td>
<td>0 0.5 1 1.5 1 1</td>
<td>0 0.5 1 1.5 1 1</td>
</tr>
</tbody>
</table>

Figure 4 VPA effect on topoisomerase II α mRNA expression. RT-PCR for topoisomerase II (Topo II) α- and β-actin (β-act) in CAL-62 cells treated with 0.5, 1, or 1.5 mM VPA for 24, 48, and 72 h.
treated with VPA alone. Instead, the present findings show that VPA increases the killing efficiency of doxorubicin about threefold in CAL-62 cells and about twofold in ARO cells; this is achieved with VPA at a dose of 0.7 mM, which is without any serious side effects and corresponds to the plasma levels reached in patients treated for epilepsy. Previous reports show that other HDAC inhibitors, such as trichostatin A (Greenberg et al. 2001a), sodium butyrate (Greenberg et al. 2001b), and suberoyl anilide hydroxamic acid (SAHA) (Mitsiades et al. 2005) are able to suppress cell growth of thyroid cancer cell lines and sensitize them to cytotoxic chemotherapy (Massart et al. 2005, Mitsiades et al. 2005, Rho et al. 2005), but the side effects of these drugs are still under evaluation.

The mechanisms underlying doxorubicin’s cytotoxicity include apoptosis induction and cell cycle arrest. We demonstrate that VPA treatment notably increases apoptosis induction by doxorubicin, by enhancing caspase 3 activation. The potentiation of apoptosis could be due to VPA’s HDAC inhibitory effect. Acetylation of histones, which is a very early event, has been widely reported to lead to conformational changes in DNA and chromatin decondensation (Lindemann et al. 2004, Dokmanovic & Marks 2005), which appears after 24–48 h of HDAC inhibitor treatment (Marchion et al. 2004, 2005). Marchion et al. (2005) recently showed that VPA is able to potentiate apoptosis induced by epirubicin and aclarubicin in estrogen-responsive breast cancer cells MCF-7, and demonstrated that this effect is linked to an increased interaction between the DNA and the drug. We suggest that histone hyperacetylation, by inducing a more open structure of chromatin, may promote a better binding of the drug to the relaxed chromatin DNA, thus the same amount of nuclear doxorubicin, as resulted in our experiments on doxorubicin uptake, would have an increased effect. Our results are in agreement with a report by Niitsu et al. (2000) that AN 9, a derivative of butyric acid, potentiates the cytotoxicity of doxorubicin, without affecting either intracellular or nuclear uptake of the drug.

The second mechanism by which doxorubicin exerts its cytotoxic effect is through its interaction with the DNA–topoisomerase II complex. This interaction has been regarded as a primary triggering event for the growth arrest induced by doxorubicin (Gewirtz 1999). Topoisomerase II inhibitors, like doxorubicin, act by trapping topoisomerase II in a covalent complex with DNA. These ‘cleavable complexes’ can act as physiological barriers to DNA replication, resulting

![Figure 5](image)

**Figure 5** VPA effect on topoisomerase II α protein. CAL-62 cells were treated with 0.5–1.5 mM VPA for 72 h; protein expression was analyzed by western blot using an antibody for topoisomerase II α (170 kDa). Equal loading and transfer were verified by reprobing the membranes with an actin antibody.

### Effect of VPA on free radical generation

ROS production is one of the mechanisms proposed for doxorubicin’s cytotoxic action, but it is also especially related to systemic toxicity and in particular to cardiac damage (Minotti et al. 2004), so we evaluated whether VPA was able to modify the levels of these toxic compounds. As reported in Fig. 6, ROS levels after the combined treatment with VPA and doxorubicin were not higher than those observed after doxorubicin exposure alone, suggesting that this pathway is not involved in potentiating the effects of doxorubicin.

### Discussion

This study demonstrates that, in ATC cell lines, VPA increases the killing efficiency of doxorubicin at least twofold. We have reported elsewhere that VPA, at a concentration of 1 mM, induces cell re-differentiation (Fortunati et al. 2004) and apoptosis (Catalano et al. 2005) in poorly differentiated thyroid cancer cells. The former effect depends on the ability of VPA to restore NIS expression and its membrane localization; the latter is related to the VPA-induced up-regulation of p21 and down-regulation of cyclin A, at both mRNA and protein levels.

Here, we demonstrate that, unlike what occurs in poorly differentiated thyroid cancer cells, VPA is unable to induce apoptosis in anaplastic thyroid cancer cells. We previously showed, in the same cell lines, that VPA promotes NIS expression but fails to target the iodine symporter correctly to membranes (Fortunati et al. 2004). Taken together, these data indicate that anaplastic thyroid cancer cannot be adequately treated with VPA alone. Instead, the present findings show
in apoptosis. Topoisomerase II inhibitors delay the G₂/M transition (Mikhailov et al. 2004) and in several systems it appears that the accumulation of cells with 4n DNA content acts as a precursor to cell death (Johnstone & Licht 2003). In the present study, we show that VPA markedly enhances the cell cycle arrest in G₂/M induced by doxorubicin treatment. The better binding of topoisomerase II to the relaxed chromatin DNA might explain the strongly enhanced cell population in the G₂/M phase, a situation that is closely linked to the observed induction of apoptosis. The same phenomenon has been reported in lung carcinoma cells treated with doxorubicin and AN 9 (Niitsu et al. 2000).

The ability of HDAC inhibitors to modulate topoisomerase II levels has been suggested as an additional mechanism for drug enhancement. It has been reported that sodium butyrate confers hypersensitivity to etoposide in human leukemic cells through induction of topoisomerase II expression (Kurz et al. 2001). Moreover, in related work, trichostatin A (TSA; another HDAC inhibitor) was shown to induce the activity of the topoisomerase II α gene promoter in mouse 3T3 fibroblasts (Adachi et al. 2000). In contrast to these explanations, our results failed to demonstrate any change in topoisomerase II levels both at mRNA and protein level in explanations, our results failed to demonstrate any change in topoisomerase II levels both at mRNA and protein level in

In conclusion, VPA, a clinically available HDAC inhibitor, notably increases apoptosis and cell cycle arrest induced by doxorubicin in anaplastic thyroid cancer cell lines. We are aware that the results obtained in cell lines must be validated in vivo models. Taking into account these limits, these results might offer a rationale for clinical studies of a new combined therapy, in an effort to improve the clinical outcome of patients with anaplastic thyroid cancer.

Funding

This study was supported by the Special Project ‘Oncology’, Compagnia San Paolo, Turin, by MIUR, and by Regione Piemonte. R. M. was supported by a grant from Associazione Bossolasco. The authors declare that there is no conflict of interest that would prejudice the impartiality of the reported research.

References


www.endocrinology-journals.org
Haigh PI 2000 Anaplastic thyroid carcinoma. *Current Treatment Options in Oncology* 1 353–357.
Haigh PI, Ituarte PH, Wu HS, Treseler PA, Posner MD, Quivey JM, Duh QY & Clark OH 2001 Completely resected anaplastic thyroid carcinoma combined with adjuvant chemotherapy and irradiation is associated with prolonged survival. *Cancer* 91 2333–2342.
Slesinger PA & Singer HS 1987 Effect of anticonvulsants on cell growth and enzymatic and receptor binding activity in neuroblastoma X glioma hybrid cell culture. *Epilepsia* 28 214–221.

Received in final form 19 July 2006
Accepted 7 August 2006
Made available online as an Accepted Preprint 12 September 2006