Lignans and Norlignans Inhibit Multidrug Resistance Protein 1 (MRP1/ABCC1)-mediated Transport

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Abstract. *Background: Multidrug resistance protein 1 (MRP1/ABCC1) is one of the drug efflux pumps mediating multidrug resistance in several cancer types. Efficient nontoxic inhibitors of MRP1-mediated transport are sought to potentially sensitise cancer cells to anticancer drugs. This study examined the potency of a series of plant lignans and norlignans of various structures to inhibit MRP1-mediated transport from human erythrocytes. The occurrence of MRP1 in the human erythrocyte membrane makes this cell a useful model in searching for efficient MRP1inhibitors. Materials and Methods: The inhibition of 2',7'-bis-(carboxypropyl)-5(6)-carboxyfluorescein (BCPCF) transport from human erythrocytes was measured fluorymetrically. In order to study possible membrane-perturbing effects of lignans and norlignans, the potency of these compounds to induce haemolysis, erythrocyte shape change, and phosphatidylserine (PS) exposure in the external layer of the erythrocyte membrane was examined. Results: Nine compounds (six norlignans and three lignans) of the fourteen that were tested inhibited BCPCF transport from human erythrocytes. The most efficient inhibitor, the norlignan coded L1, had IC_{50}=50 μM. Structure–activity relationship analysis showed that the strongest inhibitors were found among lignans and norlignans bearing a carbonyl function at position C-9. The highly oxidised structures and the presence of an ionisable group such as the carboxylic acid function enhance activity. All compounds that significantly decreased BCPCF transport were non-haemolytic, did not cause PS exposure and did not have any effect on erythrocyte shapes up to 200 μM. Conclusion: Lignans and norlignans can inhibit MRP1-mediated transport from human erythrocytes and should be further investigated as possible agents reversing multidrug resistance.*

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Key Words: Human erythrocyte, BCPCF, phenolic compound, haemolysis, lignans, norlignans, multidrug resistance, MRP1/ABCC1.

Lignans are a heterogeneous group of naturally occurring phenolic compounds that have attracted much attention because of their broad range of biological activity, including anti-oxidative (1), anti-proliferative (2, 3), apoptotic (2-5) and anticancer (3, 6-9) effects. As they occur in oil seeds, vegetables, grains, berries, and fruits, lignans are part of the normal human diet and are studied widely due to their health-promoting properties. Lignans are extracted from various plants, including several tree species, which constitute a rich source of these compounds (10, 11).

Cancer multidrug resistance (MDR) is one of the major causes of failure of clinical chemotherapy. MDR is often associated with an adenosine-5'-triphosphate (ATP)-dependent decrease in cellular drug accumulation mediated by MRP1 (ABCC1) and/or P-glycoprotein (P-gp, ABCB1) belonging to the ATP-binding cassette (ABC) superfamily of transport proteins (12-14). Nontoxic inhibitors able to block the transport function of MRP1 and/or P-gp proteins might be potentially used to overcome MDR. Flavonoids and stilbenes, plant polyphenols commonly found in vegetables and fruits, have been shown to modulate different ATP-binding cassette transporters, including MRP1, in several cell types (15-20). However, there are only a few reports concerning the effect of individual lignans on MDR associated with MRP1 (21-23). Therefore, the present study investigated a series of fourteen lignans and norlignans of various structures for their ability to inhibit MRP1-mediated transport through the human erythrocyte membrane. The occurrence of MRP1 and lack of P-gp in the human erythrocyte membrane makes this cell useful in searching for efficient MRP1 inhibitors. In order to study possible membrane-perturbing (toxic) effects of lignans and

norlignans, the potency of these compounds to induce haemolysis, erythrocyte shape change, and phosphatidylserine (PS) exposure in the external layer of the erythrocyte membrane was examined in parallel.

Materials and Methods

Chemicals. The 9-norlignans L1 and L4 were semi-synthetically prepared as described previously (24). L2, L3, L5, L6, and L7 were prepared by further synthetic modifications of L1. L2 was obtained by hydrogenation of L1 with Pd/C and H_2 in ethanol. L3 was prepared by acid catalyzed esterification of L2 in methanol/H_2SO_4. L5 was obtained by treatment of L1 in trifluoroacetic acid (TFA) and L6 was obtained by reduction of L5 with lithium aluminium hydride (LAH) in dry tetrahydrofuran (THF). L7 was prepared from L5, first by esterification (as above) followed by oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in dichloromethane. When necessary, the prepared compounds were purified by column chromatography and analyzed by nuclear magnetic resonance spectroscopy (NMR), gas chromatography-mass spectrometry (GCMS) and high resolution mass spectrometry (HRMS). L10 (7-hydroxymatairesinol) and L12 (pinoresinol) were isolated from Norway spruce (*Picea abies*) knotwood material (10, 11). The lignans L8 (matairesinol), L9 (conidendrin), L11 (7-oxomatairesinol), and L13 (7-hydroxy-secoisolariciresinol) were prepared by synthetic modification of L10 as described previously (25-27). Lignan L14 (4,4'-dihydroxy-enterolactone) was prepared by demethylation of L8. The purity (>95%) and the structure of all lignans were analysed by GCMS and NMR. The chemical characteristics of norlignans (L1-L7) and lignans (L8-L14) are presented in Figure 1. The tested compounds were dissolved in DMSO. 2',7'-*Bis*-(carboxypropyl)-5-(6)-carboxyfluorescein acetoxymethyl ester (BCPCF-AM) was obtained from Molecular Probes (Eugene, USA) and FITC-Annexin V was obtained from BenderMed Systems (Vienna, Austria).

Isolation of erythrocytes. Blood drawn from the authors (M.B-H., A.W., and H.H.) by venipuncture into heparinised tubes was centrifuged (10 min, 3000 × *g*) and the buffy coat was removed. The erythrocytes (1.65×10^9 cells/ml) were suspended in buffer of pH 7.4 containing Hepes (10 mM), NaCl (150 mM), KCl (5 mM), $CaCl_2\bullet2H_2O$ (1.8 mM), $MgCl_2\bullet6H_2O$ (1 mM), and glucose (10 mM) and were stored at +4˚C and used within 36 h.

Measurement of BCPCF efflux. Erythrocytes (3.3×10^8 cells/ml) were loaded with BCPCF-AM (2 μM) for 30 min at 37˚C. Following three washes on ice, the erythrocytes (1.65×10^8 cells/ml) were incubated at 37˚C for 60 min with or without the tested lignan or norlignan (10, 50, 70, 100, 150, and 200 μM). Following incubation, the samples were centrifuged (1 min, 3000 × *g*, 0˚C), then the extracellular BCPCF fluorescence intensity was measured using 96-well plates on a PerkinElmer Wallac VICTOR^2 1420 Multilabel Counter (Turku, Finland) at λ_{ex}=485 nm and λ_{em}=535 nm. The inhibition of BCPCF transport (I) was calculated from the formula $I=(F_C–F_L/F_C)\times100\%$, where F_C is the fluorescence of the control supernatant and F_L the fluorescence of the supernatant from a sample incubated with the tested compound. The IC_{50} values, *i.e.* the concentration of compound required to obtain 50% inhibition of BCPCF efflux, were determined from dose–response curves obtained from three to four separate experiments performed in duplicate with erythrocytes from different blood donors.

Haemolytic activity. The degree of haemolysis was determined by comparing the amount of haemoglobin in the supernatant against a standard curve. The absorbance of supernatants was measured in parallel with fluorescence on a PerkinElmer Wallac VICTOR^2 1420 Multilabel Counter (Turku, Finland) at λ=560 nm.

Erythrocyte shape and PS exposure. To study the shape changes induced by lignans L9, L11, L14 and norlignans L1, L2, L5, L7 (100 and 200 μM), the erythrocyte samples were put onto glass coverslips, inverted, and immediately examined with a phase contrast microscope at a magnification of ×500. Following pre-treatment of the erythrocytes with the aminophospholipid translocase inhibitor *N*-ethylmaleimide (10 mM, RT, 30 min, two washes) and treatment with lignans or norlignans (100 μM, 37˚C, 60 min), the cell suspensions were labelled with FITC-Annexin V and analysed for PS exposure with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Statistical analysis. The final results are presented as the mean±SE of n separate experiments. Differences between means were evaluated by Student's *t*-test. The values were taken as significantly different when $p\leq0.05$.

Results

Nine compounds (six norlignans and three lignans) of the fourteen tested (coded L1-L14, Figure 1) inhibited BCPCF transport from human erythrocytes (Figure 2). The most efficient inhibitors were L1 (IC_{50}=50 μM), L7 (IC_{50}=100 μM) and L14 (IC_{50}=125 μM). The IC_{50} values for L1, L7 and L14 were determined from dose–response curves (Figure 3). For six compounds (L2, L4, L5, L6, L9 and L11), the inhibition of BCPCF transport was lower than 50% at concentrations ≤200 μM. All the tested lignans and norlignans were non-haemolytic up to 200 μM and did not cause PS exposure (data not shown). Compounds that significantly decreased BCPCF transport (besides L1, L7, L14 also L2, L5, L9) did not have any effect on erythrocyte shape up to 200 μM.

Discussion

It has previously been shown that natural phenolic compounds, for example flavonoids and stilbenes, are interesting as potential agents to inhibit MRP1-mediated transport (15-20). Using human erythrocytes as a model, a large variety of different compounds have been previously tested as potential MRP1 inhibitors (18-20, 28-30). By studying the structure-activity relationship, it was previously shown that the strongest inhibitors among the flavonoids were flavanones bearing a hydrophobic group at position 8C (19) and that among stilbenes, the increase in resveratrol inhibitory activity positively correlated with the oligomerisation state of the compound (18). Plant lignans, another group of naturally occurring phenolic compounds, have long been known to have beneficial biological properties, but only a few reports concerning the role of plant lignans and their derivatives in

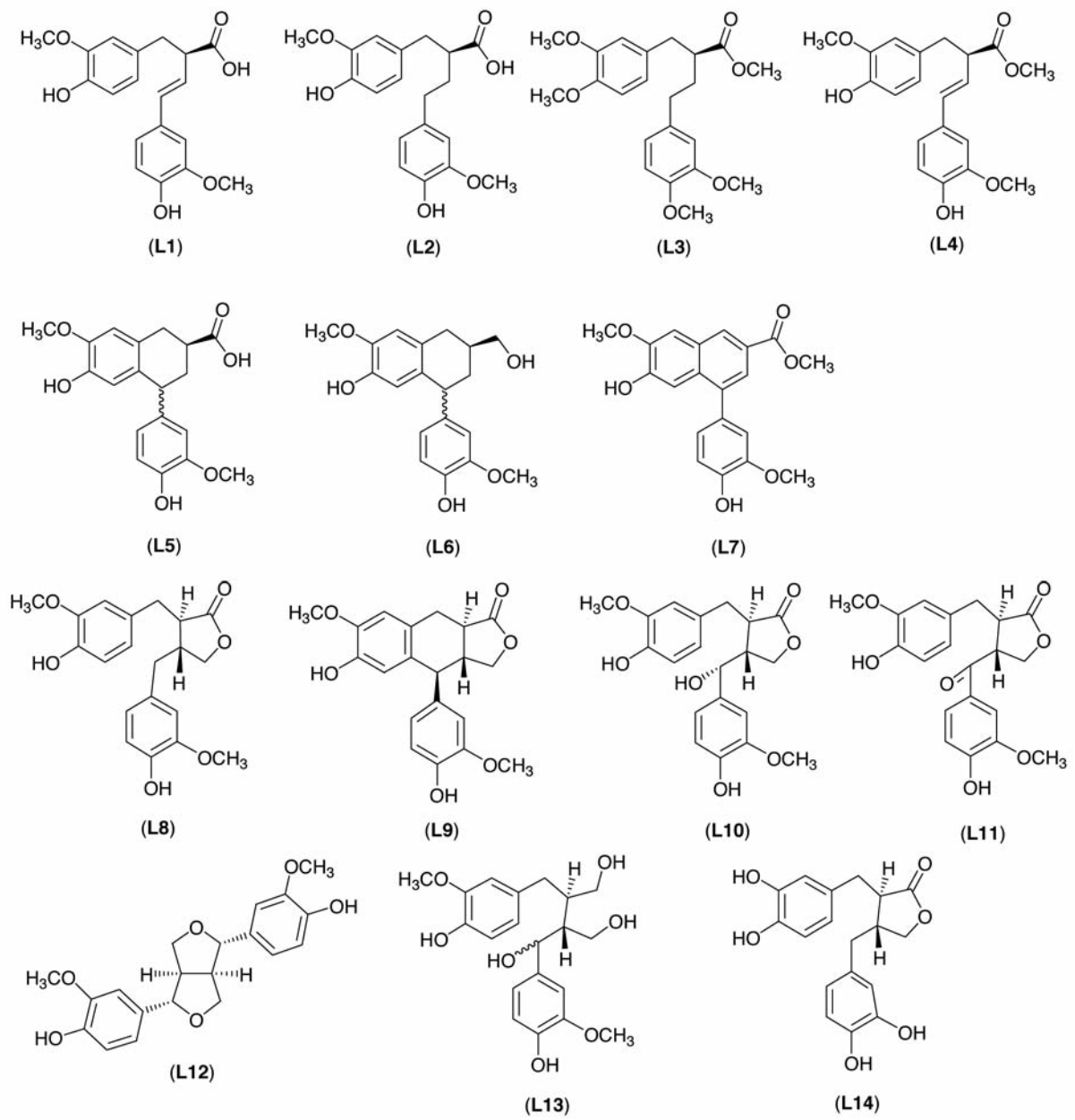

Figure 1. *Structures of lignans and norlignans*.

reversing multidrug resistance have been published (21-23, 31-35). This study of fourteen lignans and norlignans with various chemical structures shows that these phenolic compounds can inhibit MRP1-mediated transport from human erythrocytes. The most efficient inhibitors were L1 (IC_{50}=50 μM), L7 (IC_{50}=100 μM) and L14 (IC_{50}=125 μM). The inhibitory effect of L1 was clearly evident even at the low micromolar range. Compared with structurally related polyphenols previously studied in the erythrocyte system (18, 19), the IC_{50} values of the effective lignans and norlignans were not particularly low (compared with the resveratrol derivative (+)-α-viniferin (IC_{50}=0.8 μM) and the flavonoids euchrestaflavanone A and sophoraflavanone H (IC_{50}=3 μM)). Importantly however, all the tested lignans and norlignans were non-haemolytic and did not have a significant effect on the transmembrane PS distribution or erythrocyte shape up to 200 μM, which suggests a favourable 'therapeutic width', the necessary and crucial property for drugs to be used as MRP1 inhibitors. Structure–activity relationship analysis showed that the strongest inhibitors were found among lignans and

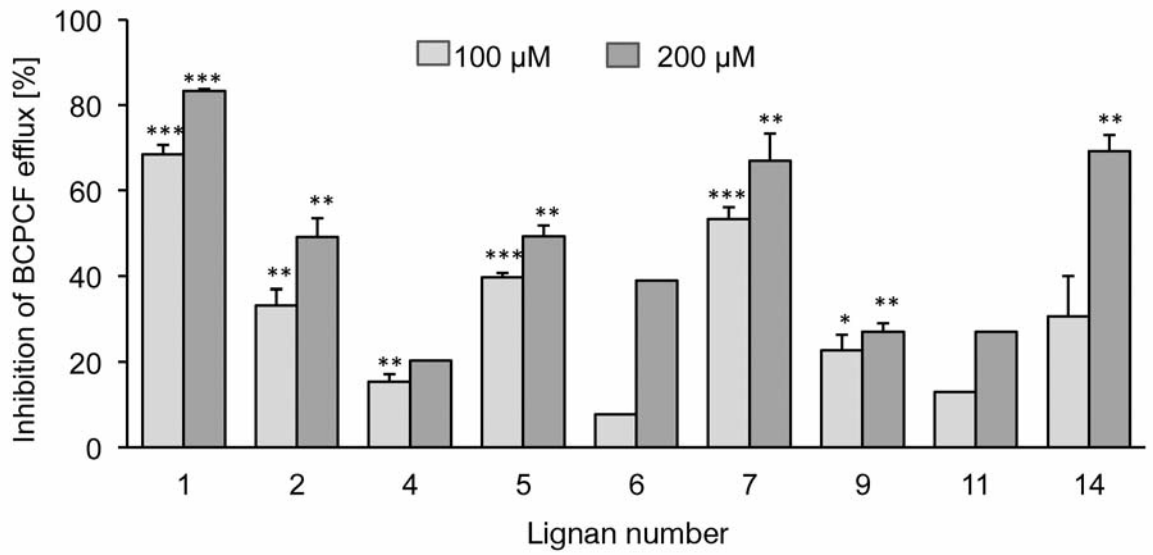

Figure 2. *Inhibition of BCPCF efflux from human erythrocyte caused by various lignans and norlignans. Compounds are numbered according to Figure 1. Erythrocytes were treated with lignans or norlignans for 60 min at 37°C. Data are the mean±SE of duplicate determinations in three to four experiments or the mean of duplicate determinations of two experiments. Only results for compounds showing inhibition greater than 10% are presented. *p<0.05, **p<0.01, ***p<0.001.*

norlignans bearing a carbonyl function at position C-9. Comparing the most efficient inhibitor L1 with the compound L2, it is clear that the lack of a double bond between C-7' and C-8' in L2 decreases the inhibitory potency approximately two times. The replacement of the free carboxylic acid with the methyl ester at C-9 in L1 leads to L4 and decreases the inhibitory potency by a factor of four. Similarly, the replacement of the free carboxylic acids with the methyl ester at C-9 in L2 and the additional methylation at the 4 and 4' positions leads to a complete disappearance of inhibition for L3. These facts stress the importance of the presence a carboxylic acid function at the C-9 position and indicate that phenolic groups contribute to the activity of norlignans. Cyclisation of L1 leads to L5 and causes an decrease in inhibitory potency. The introduction of a butyrolactone ring in L5 group leads to L9 and a decrease in inhibitory potency. Aromatisation of the ring system of L5 leads to L7 and causes a significant increase in inhibitory potency. The naturally occurring lignans matairesinol (L8), 7-hydroxymatairesinol (L10), pinoresinol (L12) and 7-hydroxysecoisolariciresinol (L13) were inactive as MRP1 inhibitors. The addition of an oxygen atom (oxidation) at the C-7 position of L8 leads to 7-oxomatairesinol (L11), with moderate inhibitory potency. However, didemethylation of L8 leading to L14 completely changes the inhibitory properties of the compound, converting an inactive lignan to a lignan with high inhibitory potency. When comparing different lignan and norlignan structures, it is evident that the number of hydroxyl groups is important but not the only factor

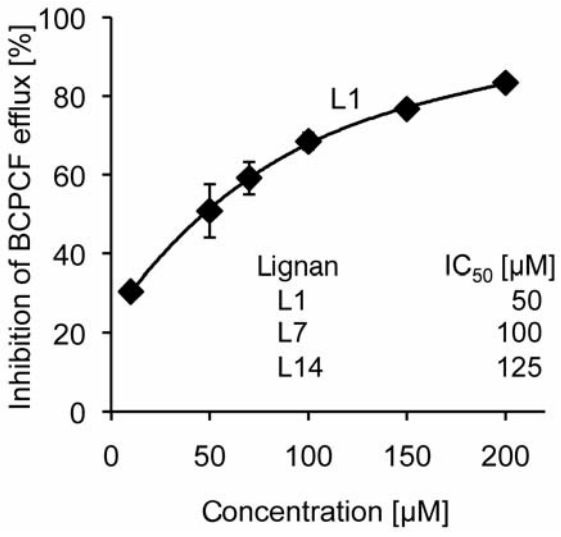

Figure 3. *Concentration-dependent inhibition of BCPCF efflux from human erythrocytes caused by L1. Erythrocytes were treated with L1 for 60 min at 37°C. Data are the mean ± SE of duplicate determinations in three experiments. Inset: IC_{50} values for L1, L7, and L14.*

determining the inhibitory properties of the compounds. The number and positions of hydroxyl groups as well as the presence of carbonyl functions, especially carboxylic acids, are important for the inhibitory potency. It seems that highly oxidised lignan and norlignan structures enhance activity. The

presence of an ionisable group, such as a carboxylic acid, gives the highest activity, which suggests that negatively charged structures (anions) may be involved in the mechanism. This is not surprising, since MRP1 effectively transports anionic substrates (28, 36-38).

Structure–activity relationship characteristics of lignans and norlignans as inhibitors of MRP1-mediated transport in human erythrocytes cannot be meaningfully related to literature data reporting the effect of lignans on other multidrug resistance phenomena associated with MRP1 because only the effect of dibenzocyclooctadiene lignans have been studied (21-23). Of these, schizandrins such as schisandrin A, schisandrin B, schisantherin A, schisandrol A, and schisandrol B are potent inhibitors of MRP1-mediated drug resistance in leukaemia cell lines overexpressing MRP1, showing concentration-dependent inhibitory effects (21). Two other dibenzocyclooctadiene lignans, deoxyschizandrin and γ-schizandrin, effectively overcome MRP1-mediated drug resistance in human lung cell carcinoma (22). The reversal effect of deoxyschizandrin and γ-schizandrin seems to be dependent on the R-biaryl configuration and the absence of a hydroxyl group at C-8, the common structural feature of both these active lignans (22). Schisandrins are an interesting group of lignans because many of them are dual inhibitors of both MRP1 and P-gp, as shown in two human leukaemia cell lines over-expressing MRP1 (21) and P-gp (35), respectively. Notably, a reversal of P-gp-mediated multidrug resistance has also been shown for the dibenzocyclooctadiene compound gomisin A (32), nordihydroguaiaretiacid derivatives M4N and maltose-M3N (33), and a derivative of podophyllotoxin L1EPO (34). Considering the fact that the MDR phenotype exhibited by cancer cells is mainly due to the ABC drug transporters P-gp, MRP1, and breast cancer resistance protein (ABCG2) (39, 40) the use of compounds with a broad spectrum of activity may be more beneficial than the use of a combination of several specific drugs. From this point of view, dual inhibitors of MRP1 and P-gp might bring advantages to clinical chemotherapy by reducing drug-drug interactions and overall toxicity. In addition to transport-inhibitory properties, antiproliferative, apoptotic, anticancer, and antioxidative effects have been reported for some lignans, for example mataresinol (5), hydroxymataresinol (8), lariciresinol (3), and phyllotoxin glucoside (2). Schisandrin B, a dual inhibitor of MRP1 and P-gp, has also been shown to enhance doxorubicin-induced apoptosis of human hepatic carcinoma and breast cancer cells without enhancing apoptosis in normal cells (4). One of the most efficient lignans selected as inhibitors of MRP1 transport in this study, L14, has been reported to be an effective radical scavenger (1). The combined impact of lignans on various biological targets might bring health benefits.

Conclusion

This study shows that lignans and norlignans can inhibit MRP1-mediated transport from human erythrocytes and suggests that this group of compounds should be further investigated as possible agents reversing multidrug resistance. To analyse the biological effects of lignans and norlignans, the importance of different chemical groups in their structure for their effects must become clear. To date, only a few lignans can be isolated in sufficient amounts for scientific research. The large-scale availability of hydroxymatairesinol by isolation from knotwood material (10, 11) makes it a potential starting compound for the synthesis of many interesting new lignans whose biological activities can be studied.

Acknowledgements

We are grateful to the Paulo Foundation, the Borg Foundation, and Understödsföreningen Liv och Hälsa r.f. for financial support.

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Received September 17, 2010
Revised October 13, 2010
Accepted October 15, 2010