Natural Product Sciences
7(2) : 38~44 (2001)

Potential Induction of Quinone Reductase Activity of Natural Products in Cultured Murine Hepa1c1c7 Cells

Yeon Hoi Heo and Sang Kook Lee*

College of Pharmacy, Ewha Womans University, 11-1 Daehyun-dong, Seodaemun-ku, Seoul 120-750, Korea

Abstract – NAD(P)H:quinone reductase (QR), known as DT-diaphorase, is a kind of detoxifying phase II metabolic enzyme catalyzing hydroquinone formation by two electron reduction pathway from quinone type compounds, and thus facilitating excretion of quinoids from human body. With the usefulness of QR induction activity assay system for the modulation of toxicants, in the course of searching for cancer chemopreventive agents from natural products, the methanolic extracts of approximately two hundreds of oriental medicines were primarily evaluated using the induction potential of quinone reductase (QR) activity in cultured murine Hepa1c1c7 cells. As a result, several extracts including *Hordeum vulgare*, *Momordica cochinchinensis*, *Strychnos ignatii*, *Houttuynia cordata*, and *Polygala japonica* were found to significantly induce QR activity. In addition, the methylene chloride fraction of *H. vulgare* , one major dietary food source, showed potent induction of QR activity (CD=6.4 μg/ml). Further study for isolation of active principles from these lead extracts is warranted for the discovery of novel cancer chemopreventive agents.

Key words – NAD(P)H:quinone reductase, natural products, murine Hepa1c1c7 cells, chemopreventive agents.

Introduction

Cancer chemoprevention has been considered as a promising approach to modulate the carcinogenic process by ingestion of dietary or pharmaceutical agents (Sporn and Newton, 1979; Hong and Sporn, 1997). A variety of chemical entities have been reported to be effective against chemical carcinogenesis in several organs, and some are candidates for inhibition of human cancer development (Kelloff *et al.*, 1992). These agents may function by a multiple action mechanisms, inhibiting, retarding or delaying at all major stages of carcinogenesis (Kelloff *et al.*, 1992; Wattenberg, 1997). One plausible mechanism involves the induction of phase II detoxification enzymes, such as QR [NADP(H):quinone oxidoreductase] and glutathione *S*-transferase (GST) (Talalay *et al.*, 1981). Several naturally occurring or synthetic compounds have been identified as potential chemopreventive agents based on the ability to induce phase II enzymes (Wattenberg *et al.*, 1986; Zhang *et al.*, 1994). Especially, sulforaphane (Zhang *et al.*, 1994), brassinin (Mehta *et al.*, 1995), withanolides (Kennelly *et al.*, 1997) and flavonoids (Cheng *et al.*, 1997; Song *et al.*, 1999) from natural products have been identified to enhance the activity of QR with cultured Hepa 1c1c7 cells.

On the basis of this information the study was undertaken to evaluate the potential of natural products, originated from oriental medicines and dietary vegetables, for induction of QR activity utilizing cultured Hepa1c1c7 cells. Approximately two hundred methanolic extracts of natural products were evaluated, and it was found that the methylene chloride fraction of *Hordeum vulgare* induced QR activity with great potentcy.

Experimental

Chemicals and Cell Cultures – β-Naphthoflavone, *tert*-butylhydroquinone, crystal violet, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), tween-20, menadione, digitonin, glucose 6-phosphate, β-NADP, FAD, dicoumarol, glucose-6-phosphate dehydrogenase, sodium dodesyl sulfate and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Cell culture media (α-MEM), fetal bovine serum (FBS) and supplements were purchased from Life Technologies, Inc. (Grand Island, NY). Hepa1c1c7 murine hepatoma cells were cultured in α-MEM with 10% FBS, 100 units/ml

*Author for correspondence.

penicillin G, and 100 μg/ml streptomycin sulfate (37°C, 5% CO_2).

Preparation of extracts of natural products – Natural products including Korean medicinal plants were purchased from herbal market (Han-Yang Yutong Co.) in Seoul, Korea. The botanical identification was performed in the Department of Pharmacy, College of Pharmacy, Ewha Womans University, Seoul, Korea, and the voucher specimen was deposited in this department. Each of dried products was sliced, and extracted with 100% methanol (x3). The methanol extracts were concentrated under reduced pressure below 40°C and stored at –20°C until use. Some methanolic extracts were partitioned with petroleum ether, *n*-hexane, methylene chloride, ethyl acetate for further study.

Quinone reductase (QR) assay – QR activity was assessed in 96-well plates with Hepa 1c1c7 murine hepatoma cells as described previously (Gerhäuser *et al.*, 1997). Briefly, the cells were plated at 5,000 cells/microtiter 96-well plate (200 μl of medium/well) and cultured for 24 h. Test samples were added in 0.5% DMSO in fresh medium. After the plates were exposed for 48 h, the media were decanted, and the cells were lysed by incubation with 50 μl of a solution containing 0.8% digitonin and 2 mM EDTA, pH 7.8. The plates were agitated on a plate shaker for an additional 10 min after the 200 μl of the reaction mixture was added to each well. A blue color developed and the reaction was stopped after 5 min by the addition of 50 μl of a solution containing 0.3 mM dicoumarol in 0.5% DMSO and 5 mM potassium phosphate, pH 7.4. The plates were then scanned at 595 nm. The protein contents of each well were determined by the crystal violet protein staining methods, and the specific activity was defined as nmol of MTT blue formazan formed per mg protein per min as described by Prochaska and Santamaria (Prochaska and Santamaria, 1988). A plot of the ratio of QR-specific activities of treated cells to control cells as a function of inducer concentration. When necessary, the CD-value (*C*oncentration required to *D*ouble the specific activity of QR) was determined to compare the relative potential. A series of concentrations of β-naphthoflavone and *tert*-butylhydroquinone were used as standard positive controls.

Results and Discussion

Previous studies have suggested that induction of phase II detoxification enzymes is considered as a relevant mechanism for cancer chemoprevention. Many compounds derived from natural products or synthetic origins, such as sulforaphane, indole-3-carbinol, sulforamate, dithiolethione, ethoxyquin, and β-naphthoflavone, have been reported to exhibit broad-based anticarcinogenic activity against a variety of chemical carcinogens at multiple target sites in animal models through induction of phase II detoxification enzymes (Zhang *et al.*, 1994; De Long *et al.*, 1985; Grubbs *et al.*, 1995; Gurtoo *et al.*, 1985), such as QR and GST. These inducible enzymes facilitate the metabolic detoxification of xenobiotics in mammals and can achieve chemopreventive activity by modification of carcinogen metabolism through increased carcinogen excretion and decreased carcinogen-DNA interactions.

In our continuing effort of searching for novel chemopreventive agents from natural products, the Hepa 1c1c7 QR assay was used to identify potent detoxification enzyme inducers because the specific activity of QR rises concomitantly with other phase II detoxification enzymes in many animal tissues in response to various chemopreventive agents (De Long *et al.*, 1985; Prochaska *et al.*, 1985). In this study, the methanol extracts of ~200 natural products were primarily evaluated for potential to induce QR activity in Hepa 1c1c7 cells. As judged in the criteria of induction of QR activity with CD <50 μg/ml, several extracts such as *Hordeum vulgare*, *Momordica cochinchinensis*, *Strychnos ignatii*, *Houttuynia cordata*, and *Polygala japonica* significantly induced QR

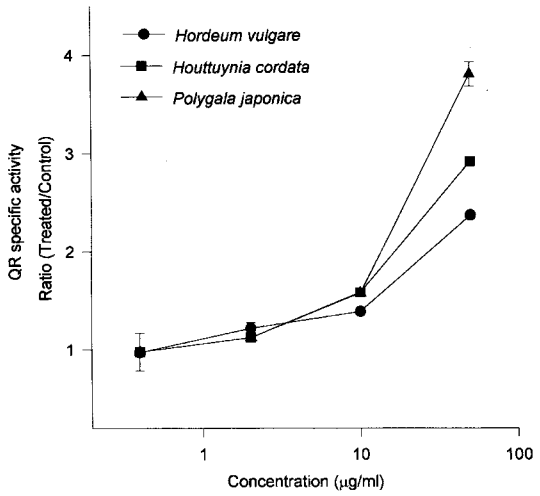

Fig. 1. Dose-response QR induction activity by active lead plant extracts.

Table 1. The effect of natural products on QR induction potential

Plant name and Authority	Family	Part used [a]	CD [b]
Acanthopanax sessiliflorum Seeman	Araliaceae	BK	>50
Achyranthes japonica Nakai	Amaranthaceae	RT	>50
Aconitum koreanum Raymond	Ranunculaceae	TB	>50
Aconitum pseudolaeve var. erectum Nakai	Ranunculaceae	RT	>50
Adenophora remotiflora Mig.	Campanulaceae	RT	>50
Agrimonia pilosa Ledeb *var. japonica* Nakai	Rosaceae	HR	>50
Ailanthus altissima (Mill) Swingle	Simarubaceae	SB	>50
Akebia quinata Dence	Lardizabalaceae	ST	>50
Albizzia julibrissin Durazz	Leguminosae	CR	>50
Alpinia katsumadai Hayata	Zingiberaceae	SD	>50
Amomum cardamomum L.	Zingiberaceae	FR	>50
Amomum xanthioides Wall	Zingiberaceae	FR	>50
Ampelopsis japonica (Thunb) Makino	Vitaceae	RT	>50
Angelica gigas Nakai	Umbelliferae	RT	>50
Arctium lappa L.	Compositae	FR	>50
Areca catechu L.	Palmae	FR	>50
Aristolochia contorta Bunge	Aristolochiaceae	FR	>50
Aristolochia debilis Sieb. et Zucc.	Aristolochiaceae	RT	>50
Artemisia apiacea Hance.	Compositae	HR	>50
Artemisia capillaris Thunb	Compositae	HR	>50
Asparagus cochinchinensis (Lour.) Merr.	Liliaceae	TB	>50
Aster tataricus L. Fil.	Compositae	RT	>50
Astragalus membranaceus (Fisch.) Bge.	Leguminosae	RT	>50
Atractylodes japonica Koidz.	Compositae	ST	>50
Benincasa hispida (Thunb.) Cogn.	Cucurbitaceae	SD	>50
Betula platyphylla Suk. *Var. japonica* Hara.	Betulaceae	CR	>50
Bletilla striata (Thunb.)Reichb. Fil.	Orchidaceae	TB	>50
Broussonetia papyrifera (L.)Vent.	Moraceae	FR	>50
Buddleia officinalis Maxim.	Loganiaceae	FL	>50
Caesalpinia sappan L.	Leguminosae	LG	>50
Caragana chamlagu Lam.	Leguminosae	LF	>50
Carpesium abrotanoides L.	Compositae	FR	>50
Carthamus tinctorius L.	Compositae	SD	>50
Chaenomeles sinensis (Thuin) Koehne	Malaceae	FR	>50
Chelidonium majus L.	Papaveraceae	HR	>50
Chrysanthemum zawadskii var latilobum Kitamura	Compositae	HR	>50
Cibotium barometz (L.) J. Sm.	Cyatheaceae	ST	>50
Cichorium intybus L.	Compositae	LF	>50
Cinnamomum cassia Blume	Lauraceae	TW	>50
Cinnamomum loureirii Nees.	Lauraceae	BK	>50
Circium japonicum DC	Compositae	RT	>50
Clematis mandshurica Rupr.	Ranunculaceae	RT	>50
Codonopsis pilosula (Franch) Nannf.	Campanulaceae	RT	>50
Corydalis ternata Nakai	Papaveraceae	TB	>50
Cremastra variabilis Nakai	Orchidaceae	TB	>50
Cucumis melo L. *var makuwa* Makino	Cucurbitaceae	PF	>50
Curculigo orchioides Garrtn.	Amaryllidaceae	ST	>50
Curcuma longa L.	Zingiberaceae	ST	>50
Curcuma zedoaria Roscoe	Zingiberaceae	ST	>50
Cuscuta chinensis Lam.	Convolvulaceae	SD	>50
Cynanchum ascyrifolium Matsumura	Asclepiadaceae	RT	>50
Cynanchum atratum Bunge	Asclepiadaceae	RT	>50
Cynanchum paniculatum Kitagawa	Asclepiadaceae	RT	>50
Cynanchum wilfordii (Max.) Hemsl.	Asclepidaceae	TB	>50
Cynomorium songaricum Rupr.	Cynomoriaceae	HR	>50
Cynthus officinalis L.	Compositae	RT	>50
Daphne genkwa Sieb. et Zucc.	Thymelaeaceae	FL	>50

Table 1. Continued

Plant name and Authority	Family	Part used [a]	CD [b]
Davallia mariesii Moore	Davalliaceae	ST	>50
Dendrobium nobile Lindl.	Orchidaceae	HR	>50
Dictamnus albus L.	Rutaceae	SB	>50
Dioscorea japonica Thunb.	Dioscoreaceae	TB	>50
Dioscorea tokoro Makino	Dioscoreaceae	TB	>50
Diospyros kaki Thunb.	Ebenaceae	LF	>50
Dipsacus japonicus Mig.	Dipsacaceae	RT	>50
Draba nemorosa L.	Cruciferae	SD	>50
Eclipta prostrata L.	Compositae	HR	>50
Ephedra sinica Stapf.	Ephedraceae	RT	>50
Equisetum hiemale L.	Equisetaceae	HR	>50
Eriobotrya japonica (Thunb) Lindl.	Malaceae	LF	>50
Eriocaulon sietoldianun Sieb. et Zucc.	Eriocaulaceae	FT	>50
Erycibe obtusifolia B.	Convolvulaceae	ST	>50
Eucommia ulmoides Oliv.	Eucommiaceae	TW	>50
Eucommia ulmoides Oliv.	Eucommiaceae	SB	>50
Eugenia caryophyllata Thunb.	Myrtaceae	SB	>50
Euphorbia lathyris L.	Euphorbiaceae	SD	>50
Euphoria longana Steud.	Sapindaceae	FR	>50
Gallus domesticus Blume	Phasianidae	ST	>50
Ganoderma lucidum Karst.	Polyporaceae	WP	>50
Gardenia jasminoides Ellis.	Rubiaceae	FR	>50
Gastrodia elata Blume	Orchidaceae	ST	>50
Ginkgo biloba L.	Ginkgoaceae	FR	>50
Glechoma longituba (Nakai) Kupr.	Labiatae	HR	>50
Gleditsia japonica var. koraiensis Nakai	Leguminosae	TN	>50
Glycine max (L.) Merr.	Leguminosae	SD	>50
Glycyrrhiza uralensis Fischer	Leguminosae	RT	>50
Hemerocallis fluva L.	Liliaceae	LF	>50
Hordeum vulgale L.	Graminae	FR	36.8
Houttuynia cordata Thunb.	Saururaceae	HR	20.6
Ipomoea hederacea Jacq.	Convolvulaceae	SD	>50
Juncus effusus L. *var. decipiens* Buchen.	Juncaceae	HR	>50
Kalopanax pictus (Thunb.) Nakai	Araliaceae	CR	>50
Kochia scoparia (L.) Schrad.	Chenopodiaceae	SD	>50
Leonurus sibiricus L.	Labiatae	SD	>50
Ligustrum lucidum Ait.	Oleaceae	FR	>50
Litchi chinensis Sonn.	Sapindaceae	FR	>50
Lycium chinensis Mill.	Solanaceae	SB	>50
Lycopus lucidus Turcz.	Labiatae	HR	>50
Magnolia denudata Desr.	Magnoliaceae	FL	>50
Magnolia officinalis Rehd. et Wils.	Magnoliaceae	CR	>50
Malva verticillata L.	Malvaceae	SD	>50
Melandrium firmum Rohrb.	Caryophyllaceae	SD	>50
Melia azedarach L. *var. japonica* Makino	Meliaceae	FR	>50
Momordica cochinchinensis Spr.	Cucurbitaceae	SD	26.9
Morinda officinalis How.	Rubiaceae	RT	>50
Morus alba L.	Moraceae	FR	>50
Morus alba L.	Moraceae	LF	>50
Morus alba L.	Moraceae	TW	>50
Mucuna birdwoodiana Tutcher.	Leguminosae	ST	>50
Nepeta japonica Max.	Labiatae	HR	>50
Oldenlandia diffusa (Willd.) Roxb.	Rubiaceae	HR	>50
Orostachys japonicus A. Berger.	Crassulaceae	HR	>50
Paeonia moutan Sims.	Paeoniaceae	SB	>50
Panax notoginseng F. H. Chen.	Araliaceae	RT	>50

Table 1. Continued

Plant name and Authority	Family	Part used [a]	CD [b]
Panax quinquefolium L.	Araliaceae	RT	>50
Paonia lactiflora Pall.	Paeoniaceae	RT	>50
Persicaria tinctoria H.Gross.	Polygonaceae	HR	>50
Phaenosperma globosa M.	Graminae	HR	>50
Pharbitis nil Choisy.	Convolvulaceae	SD	>50
Phragmites communis Trin.	Graminae	RT	>50
Phyllostachys nigra M. *var. henonis* S.	Bambusaceae	CR	>50
Phytolacca esculentum Var Houtt.	Phytolaccaceae	RT	>50
Pinus densiflora Sieb et. Zucc.	Pinaceae	TW	>50
Piper longum L.	Piperaceae	SD	>50
Polygala japonica Houtt.	Polygalaceae	HR	17.3
Polygala tenuifolia Willd.	Polygalaceae	RT	>50
Polygonum aviculare L.	Polygonaceae	HR	>50
Polygonum multiflorum Thunb.	Polygonaceae	TB	>50
Poncirus trifloliata Ratin.	Rutaceae	FR	>50
Poria cocos (S.) Wolf	Polyporaceae	ST	>50
Poria cocos (S.) Wolf	Polyporaceae	SB	>50
Poria cocos (S.) Wolf	Polyporaceae	SC	>50
Portulaca oleracea L.	Portulacaceae	HR	>50
Prunella vulgaris L. *var. lilacina* Nakai	Labiatae	HR	>50
Prunus japonica var. nakaii Rhed.	Amygdalaceae	SD	>50
Pterocarpus santalinus Lf.	Leguminosae	LG	>50
Pueraria thunbergiana Bentham	Leguminosae	RT	>50
Pueraria thunbergiana Bentham	Leguminosae	FL	>50
Pyrrosia lingua (Thunb) Far W.	Polypodiaceae	LF	>50
Rehmania glutinosa Liboschitz	Scrophulariaceae	RT	>50
Rehmannia glutinosa Liboschitz *var. purpurea* Making	Scrophulariaceae	RT	>50
Rheum coreanum Nakai	Polygonaceae	ST	>50
Rhus verniciflua Stokes	Anacardiaceae	FD	>50
Rosa laevigata Michx	Rosaceae	FR	>50
Rosa rugosa Thunb.	Rosaceae	RT	>50
Rubia akane Nakai	Rubiaceae	RT	>50
Rubus coreanus Mig.	Rosaceae	FR	>50
Salvia miltiorrhiza Bunge	Labiatae	RT	>50
Sargassum fusiforme (Harv.) Setch.	Sargassaceae	HR	>50
Schizandra chinensis Baill.	Schizandraceae	FR	>50
Scirpus yagara Ohwi	Cyperaceae	ST	>50
Scutellaria baicalensis Georgi.	Labiatae	RT	>50
Sedum albroseum Bak.	Crassulaceae	HR	>50
Selaginella tamariscina Spring	Selaginellaceae	HR	>50
Sesamum indicum DC.	Pedaliaceae	SD	>50
Siegesbeckia orientalis L. *var. pubescens* Mak.	Compositae	HR	>50
Sinomenium acutum Rehder et Wilson	Menispermaceae	ST	>50
Solanum nigrum L.	Solanaceae	HR	>50
Sophora japonica L.	Leguminosae	RT	>50
Sophora subprostrata Chun. Et. T. Chen.	Leguminosae	RT	>50
Spirodela polyrrhiza (L.) Schleid	Lemnaceae	HR	>50
Strychnos ignatii Berg.	Loganiaceae	FR	16.3
Thuja orientalis L.	Cupressaceae	FT	>50
Tribulus terrestris L.	Zygophyllaceae	FR	>50
Trichosanthes kirilowii Max	Cucurbitaceae	RT	>50
Trigonella foenum-graecum L.	Leguminosae	SD	>50
Triticum aestivum L.	Graminae	SD	>50
Typha orientalis Schum. et Thonn.	Typhaceae	PL	>50
Ulmus macrocarpa Hance	Ulmacea	FR	>50
Ulmus parvifolia Jacq.	Ulmaceae	SB	>50
Ulmus pumila L.	Ulmaceae	SB	>50

Table 1. Continued

Plant name and Authority	Family	Part used[a]	CD[b]
Uncaria sinensis Havil.	Rubiaceae	TW	>50
Undaria pinnatifida Sur.	Lamirariaceae	HR	>50
Xanthium strumarium L.	Compositae	FR	>50
Zea mays L.	Graminae	CS	>50
Zingiber officinale Roscoe	Zingiberaceae	ST	>50
Zizyphus jujuba Mill.	Rhamnaceae	FS	>50
Zizyphus jujuba Mill. *var. inermis* Rehol.	Rhamnaceae	FR	>50
β-Naphthoflavone			0.4 μM
t-Butylhydroquinone			6.2 μM

[a]Part used: HR (herb), SB (stem bark), RT (root), ST (stem), LF (leaf), TW (twig), FL (flower), FT (flower + twig), FR (fruit), SD (seed), SC (sclerotium), TB (tuber), PF (penduncle of fruit), PL (pollen), CR (cortex), FD (fluid), CS (corn silk), FS (seed in fruit), WP (whole plant), BK (bark), TN (thorn).
[b]CD (μg/ml) : Concentration required to Double the specific activity of quinone reductase.

Table 2. QR induction activity of the partion of active lead plant extracts

Plant name	Family	Part used[a]	Fraction	CD (μg/ml)[b]
Hordeum vulgare	Gramineae	FR	Hexane	24.9
			Methylene chloride	6.4
			Ethyl acetate	27.7
Houttuynia cordata	Saururaceae	HR	Petroleum ether	20.6
			Hexane	7.6
			Methylene chloride	>50
			Ethyl acetate	>50
Polygala japonica	Polygalaceae	HR	Petroleum ether	33.7
			Hexane	>50
			Methylene chloride	>50
			Ethyl acetate	>50

[a]Part used: HR (herb), FR (fruit).
[b]CD (μg/ml) : Concentration required to Double the specific activity of quinone reductase.

activity with CD values in the range of 15.0-50.0 μg/ml (Table 1 and Fig. 1). Further, based on the potent induction activity of *Hordeum vulgare*, the partion of methanol extract were performed using various solvent systems. The methylene chloride fraction of *H. vulgare* fruit extract demonstrated a remarkable induction in terms of the induction potency and low toxicity (Table 2 and Fig. 2). In addition, the hexane fraction of *Houttuynia cordata* herb extract and the petroleum ether fraction of *Polygala japonica* herb extract also potentially induced QR activity (Table 2). Since *H. vulgare* is one of major dietary food sources, the identification of active principles of QR induction will be informative for further development of cancer chemopreventive agents or study of mechanism of action mediated by the dietary source. In summary, several plant extracts shown induction potential of QR detoxification enzymes from this result might be warranted to identify the active principles and provide encouragement for further development of cancer

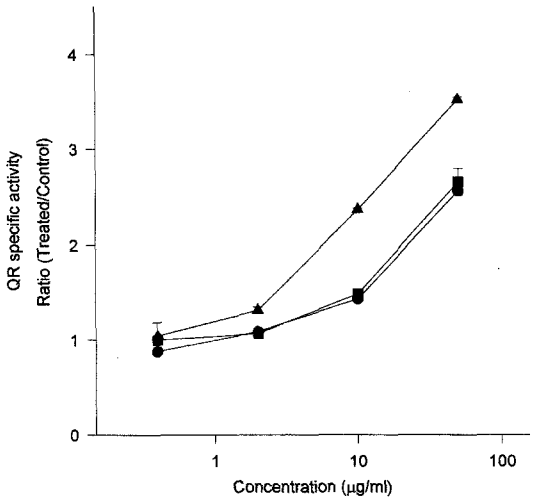

Fig. 2. Induction potential of QR activity mediated by the fraction of *H. vulgare*. Ethyl acetate fraction (●), hexane fraction (■), and methylene chloride fraction (▲) of *H. vulgare* were tested for QR activity in cultured Hepa1c1c7 cells.

chemopreventive agents from natural products.

Acknowledgements

This work was supported by the Faculty Research Fund of the Research Institute of Pharmaceutical Science, College of Pharmacy, Ewha Womans University (Fall, 1999).

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(Accepted May 23, 2001)