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Design and Evaluation of Folate-appended Methyl-β-Cyclodextrin as an Active Pharmaceutical Ingredient for Cancer Treatment*

Chemotherapy is expected to destroy the tumor cells with maximum treatment efficacy, while minimizing side effects to normal tissues. However, in the application of conventional anticancer agents, there are some unexpected limitations such as poor distribution in tumor sites, impairment of normal tissue, and lack of target specificity. In order to overcome these drawbacks, the various techniques of drug delivery for tumor cells have attracted considerable attention. To provide an active targeting-ability to a drug carrier, chemical modification by tumor targeting ligands is known, such as antibody, sugar, folic acid (FA), transferrin, and epidermal growth factor.

Recently, FA has emerged as a prominent targeting moiety capable of specific interaction with folate receptor (FR)-expressing cells [1]. FR isoform α (FR-α) consists of a high affinity folate binding protein (FBP) (dissociation constant: approximately 10^{-9}-10^{-10} M) and is expressed on plasma membrane as a glycosylphosphatidylinositol (GPI)-anchored protein [2]. FR-α is highly expressed in various human tumor cells, including malignancies of the ovary, breast, brain, lung, kidney and myeloid cells, and FR-α slightly expresses in normal tissues [3-6]. This overexpression of FR-α provides tumor cells with increased amounts of the FA essential for DNA synthesis, and seems to aid in aggressive tumor growth. Notably, the overexpression of FR-α correlates with a higher histological grade and more advanced stage of the disease in cancer patients [7]. Therefore, FR-α is one of the potent candidates, not only as an attractive marker but also a target molecule for diagnosis and chemotherapy [8]. Actually, EC145 (Vintafolide) was developed to deliver a vinca alkaloid directly to FR-α-expressing cancer cells by the introduction of FA as a tumor targeting ligand [9]. In addition, Vintafolide is being investigated in a Phase 3 study in patients with platinum-resistant ovarian cancer.

Cyclodextrins (CDs) and their hydrophilic derivatives form inclusion complexes with hydrophobic molecules. In the pharmaceutical fields, CDs are widely used for improvement of solubility, dissolution rate and bioavailability of the drugs [10,11]. Meanwhile, CDs have been reported to interact with cell membrane components such as cholesterol and/or phospholipids,

*** dr. Keiichi Motoyama, the first author of this article, got the Prof. Szejtli Prize in 2014**

resulting in the induction of hemolysis of human and rabbit red blood cells at high concentrations of CDs [12-14]. Additionally, methyl-β-cyclodextrin (MβCD) is acknowledged to disrupt the structures of lipid rafts and caveolae, which are lipid microdomains in the cell membrane, through the extraction of cholesterol from the microdomains [15]. Furthermore, we demonstrated that dimethyl-β-cyclodextrin (DMβCD) induced apoptosis through the impairment of PI3K-Akt-Bad pathway, leading to cholesterol depletion from lipid rafts in NR8383 cells, a rat alveolar macrophage cell line [16]. Notably, Grosse *et al*. reported that intraperitoneal injection of MβCD showed signs of antitumor activity in human tumor xenografted athymic nude mice [17]. However, parenteral application of MβCD is not allowed in humans [18], because of its lack of tumor cell-selectivity.

Recently, in an attempt to confer a tumor-selective cytotoxic activity to MβCD, we newly fabricated folate-appended MβCD (FA-MβCD) with average degree of substitution (DS) of folate and methyl moieties of 1.0 and 12.2, respectively [19]. The advantages of FA-MβCD as an anticancer agent are indicated as follows, compared to antibody drugs: 1) the physicochemical stability is high, 2) the batch difference in bioactivity does not occur as it is a chemically synthesized product, 3) the pharmacokinetics after intravenous administration is rarely affected by serum proteins due to its low molecular weight compound, and 4) the cost performance is superior to that of biosynthesis products. In this short review, we introduce the potential of FA-MβCD as an active pharmaceutical ingredient (API) for cancer treatment.

In vitro **antitumor activity of FA-MβCD**

To clarify the FR-α-selective antitumor activity of FA-MβCD, we evaluated antitumor activity of FA-MβCD in KB cells (FR-α (+)) and A549 cells (FR-α (-)) [19,20]. FA-MβCD displayed potent antitumor activity, compared to MβCD in KB cells, but not in A549 cells. In contrast, DMβCD showed significant antitumor activity in both KB cells and A549 cells. Additionally, in Colon-26 cells (FR-α (+)), FA-MβCD showed potent antitumor activity, compared to MβCD. Meanwhile, the antitumor activity of FA-MβCD was significantly attenuated in FR-α knockdown-KB cells produced by treatment with FR-α siRNA. These results suggest that FA-MβCD has FR-α-expressing cell-selective antitumor activity.

FA-MβCD induced apoptosis-independent cell death

In spite of the development of impressive treatment, few options for cancer cells are available. A number of promising agents with multiple mechanisms of action are under investigation. Recent studies exploring the cell death machinery have led to the discovery of alternative pathways for modulating cell death and also novel compounds inducing cancer cell demise [21]. Among cell death mechanisms, apoptotic cell death plays essential roles in cell survival, growth and tumorigenesis. MβCD is often used to disrupt lipid rafts because of its ability to deplete cholesterol stores on cell membranes. A number of studies have also demonstrated that MβCD can harm cancer cells and cause cell death by the disruption of lipid rafts. For example, cholesterol depletion by MβCD induced apoptosis and caveolae internalization in human epidermoid carcinoma cells [22]. Furthermore, we previously revealed that DMβCD elicited apoptosis through the impairment of the PI3K-Akt pathway, resulting from cholesterol depletion from lipid rafts in NR8383 cells [16]. We also confirmed that DMβCD induced apoptosis in KB cells, probably due to the cholesterol depletion, leading to a decrease in not only DNA content but also mitochondrial transmembrane potential. Actually, FA-MβCD released significant amount of cholesterol from KB cells and A549 cells to culture medium, compared to that of DMβCD. However, FA-MβCD caused cell death without lowering the DNA content and mitochondrial transmembrane potential and also activation of caspase 3/7 [23], indicating that apoptosis is not involved in cell death induced by FA-MβCD in KB cells (FR-α (+)) . Additionally, FA-MβCD did not induce cell death in A549 cells (FR-α (-)) even through its potent cholesterol depletion ability, compared to the other βCDs, under the present experimental conditions. Meanwhile, MβCD induced apoptosis in A549 cells (FR-α (-)) through not only lowering DNA content but also reducing mitochondrial transmembrane potential. Collectively, these results suggest that the extraction of cholesterol from plasma membranes by FA-MβCD is not associated with the induction of cell death.

FA- MβCD induces autophagy in cancer cells

Autophagy is a normal physiological process in the body that deals with destruction of cells in the body, and can kill the cells under certain conditions. There are several reports on autophagy or autophagic cell-death activated in cancer cells after treatment with various anticancer drugs [24]. Next, we examined whether autophagosome formation in KB cells is elicited by FA-MβCD, using Cyto-ID® Autophagy Detection Kit, which detects autophagic vacuoles in cells. The autophagic vacuoles in KB cells were observed after treatment with FA-MβCD for 2 h [23]. Additionally, the autophagic vacuoles elicited by the treatment with FA-MβCD were overwhelmingly decreased by the pretreatment with LY294002, an autophagy inhibitor. These results suggest that FA-MβCD induced the formation of autophagic vacuoles in KB cells (Fig. 1).

The dysfunctional mitochondria are recognized and degraded within cells by both nonselective autophagy and mitophagy, a selective type of autophagy. We found that FA-MβCD significantly enhanced the mitochondrial membrane potential in KB cells, indicating the induction of mitochondrial stress. Therefore, we examined the involvement of mitophagy in cell-death caused by mitochondrial stress after treatment with FA-MβCD [23]. The autophagic vacuoles and mitochondria, stained by Cyto-ID® Autophagy Detection Kit and rhodamine 123, respectively, were partially colocalized in KB cells after treatment with FA-MβCD. Therefore, these results suggest that the autophagic cell-death induced by FA-MβCD could be associated with mitophagy elicited by a mitochondrial stress (Fig. 1).

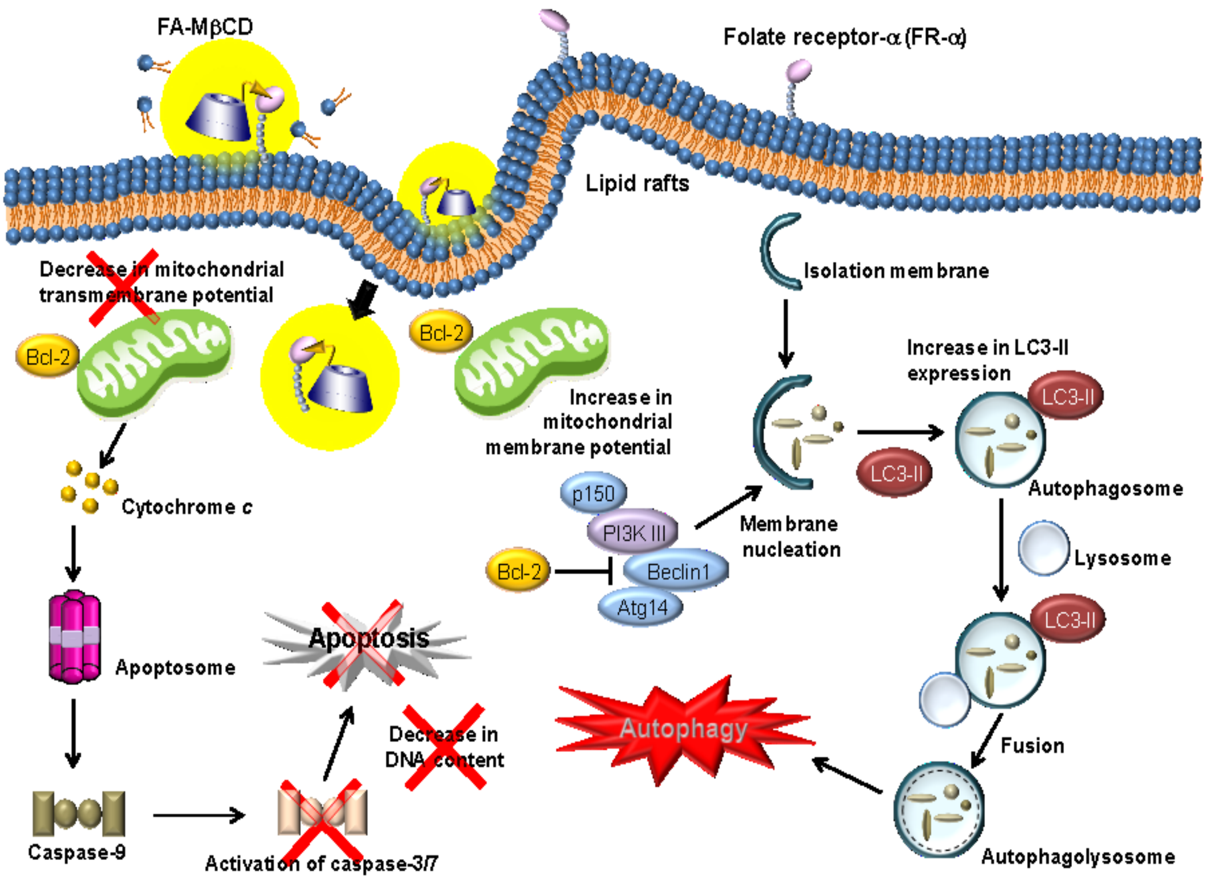

Fig. 1.: Proposed Mechanism of Antitumor Activity of FA-MβCD

In vivo **antitumor activity of FA-MβCD**

To investigate antitumor activity of FA-MβCD *in vivo*, we injected FA-MβCD solution intravenously to tumor-bearing mice. An intravenous injection of doxorubicin or MβCD slightly suppressed the tumor growth. Remarkably, FA-MβCD drastically inhibited the tumor growth after an intravenous injection [20]. Furthermore, the tumor inoculated subcutaneously completely disappeared after treatment with FA-MβCD. Surprisingly, all of the tumor-bearing mice after intravenous injection of FA-MβCD survived for at least 140 days without any relapse, while the mice treated with doxorubicin and MβCD died of sickness within 70 days.

Additionally, the body weight of mice after an intravenous injection of FA-MβCD was increased slightly as the time passed, suggesting that FA-MβCD does not have any significant side effect. In terms of blood chemistry data, doxorubicin tended to elevate the alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) values, and MβCD significantly increased blood urea nitrogen (BUN), aspartate aminotransferase (AST) and LDH levels, compared to control, suggesting induction of systemic side effects of doxorubicin and MβCD. Strikingly, no significant changes in the blood chemistry values such as creatinine (CRE), BUN, AST, ALT and LDH were observed 24 h after an intravenous injection of FA-MβCD, compared to control (5% mannitol solution) at the same dose as doxorubicin and MβCD. These results strongly suggest that FA-MβCD has the potential as a novel antitumor agent with negligible systemic side effects even after intravenous injection.

Conclusions

In conclusion, we evaluated the potential of FA-MβCD as a novel anticancer agent *in vitro* and *in vivo*. FA-MβCD provided potent antitumor activity *in vitro*, compared to MβCD in KB cells (FR-α (+)), but not in A549 cells (FR-α (-)). Furthermore, FA-MβCD drastically inhibited tumor growth after an intravenous injection to tumor-bearing mice, compared to doxorubicin and MβCD, without any significant change in blood chemistry values after an intravenous administration. These results strongly suggest that FA-MβCD has the potential as an API for cancer treatment.

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Keiichi Motoyama[1], Taishi Higashi[1] and Hidetoshi Arima[1,2,*]
[1] *Department of Physical Pharmaceutics,*
Graduate School of Pharmaceutical Sciences,
Kumamoto University,
5-1 Oe-honmachi, Chuo-ku, Kumamoto 862-0973, Japan

[2]*Program for Leading Graduate Schools "HIGO (Health life science: Interdisciplinary and Glocal Oriented) Program",*
5-1 Oe-honmachi, Chuo-ku, Kumamoto 862-0973, Japan

[*]*Corresponding author:* Hidetoshi Arima
TEL: +81-96-371-4160; fax +81-96-371-4420,
E-mail address: arimah@gpo.kumamoto-u.ac.jp

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Amylase, Halophilic Alkalithermophilic, Wadi an Natrun

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Profen, Affinity Capillary Electrophoresis, Complexation Constants, Acid Dissociation Constants, Simulations, β-cyclodextrin, TRIMEB, NMR, Electromigration Dispersion

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β-Cyclodextrin, 2,6-Dinitroaniline, Inclusion Complex, Patch Dock Server

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Functional, Light-responsive, Polymers, Supra-amphiphiles

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Diclofenac, NSAID, Skin, Delivery, Formulation, Salts

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Chiral Stability, Chiral Stationary Phase, Liquid Chromatography, Validation, Voriconazole

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Folic Acid, Nanoparticles, Carboxymethyl-β-cyclodextrin, 5-Fluorouracyl, Apoptosis, Targeting Agent

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Cyclodextrin-containing Polymer, Star Polymer, 2-(Dimethylamino)ethyl methacrylate, pH-Response, Cellular Uptake, Anti-tumor Drug Delivery, Poly(ethylene glycol), Doxorubicin, HeLa and HepG2 Cancer Cells

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Central Nervous System, Blood–brain Barrier, Drug Delivery, Transport

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Electropermeabilization, Gene Electrotransfer, Get, PlasmidDNA, Endocytosis, Muscle, RAMEB

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PEGylation, Aggregation, Cationic Nanoparticle, Pharmacokinetics, Post-modification, siRNA Delivery

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Nanoparticles, Cancer, RNAi, siRNA, EPReffect, Clinical Translation

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Antihyperlipidemic Therapy, Cardiovascular, Molecular Drug Targets, In vitro Assay, Lipoproteins, Lipid Metabolism

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Apoptosis, Caveolae, Ischemia and Reperfusion, Sphingosine-1-phosphate

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Neutrophils, Neutrophil Extracellular Traps, RAMEB, NETosis, Sphingomyelinase, NADPH-oxidases

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Membrane Domain, Lipid Raft, Subcellular Traffic, Sorting, Endocytosis

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Acidic Ca^{2+} stores, Lysosomes, Caveolae, Endothelial Cells, Insulin Resistance, Diabetes

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Arachidonic acid, Eicosanoid, Oxylipin, Inflammation

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Cholesterol, Lipid Membrane, Order Parameter, Domain Formation, Diffusion, Permeability

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Adenylyl Cyclase, Dopamine Receptor, Lipid Rafts, Signal Transduction, RAMEB

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GPR40, Free Fatty Acid, Internalization, Constitutive Activity, Arrestins, Recycling, RAMEB, Insulin Secretion

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Emulsion, Gas Chromatography, Sensory Evaluation, Propanal, 1-Penten-3-one, 1-Penten-3-ol, Hexanal, (E,E)-2,4-Heptadienal and (E,Z)-2,6-Nonadienal

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Green Coffee, Liquid Chromatography–tandem Mass Spectrometry, Molecular Modeling, Protein–polyphenol Interactions, β-Cyclodextrin

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Carboxymethylcellulose, Encapsulation, Lipid Oxidation, Maltodextrin, Pea Protein Concentrate, Spray-drying

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Fermented Beverage, Lactic Acid Bacteria, Yeast, Malto-oligosaccharides, Antioxidant Activity

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Chitosan, (2-Hydroxy)propyl-β-cyclodextrins, Carvacrol, Loading and Release, Antimicrobial Films, Glycerol

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Antimicrobial Active Packaging, Carvacrol, Chicken, Chitosan Films, Controlled Release, (2-Hydroxy)propyl-β-cyclodextrin, Unacceptable Sensory Deterioration

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Encapsulation, Delivery, Bioavailability, Nutraceuticals, Micronutrients, Nanotechnology, Nanoparticles

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Biphasic Catalysis, Hydroformylation, Rhodium, Sulfonated Phosphane, Supramolecular Chemistry. RAMEB

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Phthalates, Polyurethane Polymer, γ-cyclodextrin, Starch, Adsorption, Endocrine Disruptors, Carcinogens

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Megalo α-(1->6)-glucosaccharide, Ethyl Red, β-Cyclodextrin, Amphiphilic Surface, Azoreductase

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Adsorbents, Graphene Oxide, β-Cyclodextrin, Poly(acrylic acid), Methylene Blue, Safranine T

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CO_2 Reforming of Methane, Glucose Modified Impregnation Method, MCM-41, Syngas, β-Cyclodextrin Modified Impregnation Method

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Enhance oil Recovery, Acid Stimulation, Inclusion Complex, Clay Swelling, Response Surface Methodology

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Host-guest Recognition, Lysozyme, Magnetic Particles, Self-assembly

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PAHs, Aged Contaminated Soil, Organic Matter Quality, Sorption–desorption

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Co-production, Single-cell Biorefinery, Metabolic Engineering, Microorganism

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Biosurfactant, Desorption, Molecular Docking, Plant Uptake, Polychlorinated Biphenyls (PCBs)

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Aniline, Cr(VI), Ethylenediamine, Magnetic Graphene Oxide

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Bisphenol A, Magnetic, Molecularly Imprinted Polymers, Multiwalled Carbon Nanotube, Solid-phase Extraction, β-cyclodextrin Binary Functional Monomer, Ethylene Glycol Dimethacrylate Cross-linker

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Nanocomposites, Magnetic Hybrid Micelles, β-Cyclodextrin, Host–guest Interaction, Bisphenol A, Star-shaped Inorganic–Organic Hybrid Copolymer

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Cholesterol Sensing, Cyclic Voltammetery, Differential Pulse Voltammetery, Graphene-β-Cyclodextrin, Redox Indicator

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DDT, DDD, DDT/Fish/Sediment/Water, o,p'-DDDEF, DDA

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Chiral Separation, Cialis, Charged Cyclodextrin, Enantiomer Migration Order, NMR, Synthesis

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Neotame, Electrochemical Sensor, Cyclic Voltammetry, Differential Pulse Voltammetry

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Amino Acids, Chiral Selector, Capillary Electrochromatography, Microchip Electrophoresis, Capillary Electrophoresis–Mass Spectrometry

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Interference, Hemolysis, Lipemia, Icterus, Pediatric, Indices

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Chemically Modified Electrode, Potentiometric Sensor, Surfactant

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Allene, Vibrational Circular Dichroism, Infrared Spectroscopy, Thermodynamics, Molecular Modeling

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Pharmaceutical Analysis, Capillary Electrophoresis, Magnesium Aspartate, Chiral Separation, (2-Hydroxy)propyl-β-cyclodextrin, Laser Induced Fluorescence Detection, HPLC-fluorescence, Chiral Derivatization, o-Phthaldialdehyde, N-Acetyl-L-cysteine, Orthogonal Method

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Phenothiazines, Chiral Separation, Poly(Diallyldimethylammonium Chloride)

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Selenoamino Acid, Enantiomer, Chiral Speciation, Hyphenation Technique, Review

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Disaccharide, Noncovalent, Isomer, Epimer, Radical Migration

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Electrochemical Detection, Pulsed Electrochemical Detection, Chromatography, Capillary Electrophoresis, Microchip, Carbohydrates

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Enzymes, DNA-biosensor, Immunosensor, Enzyme Biosensor, Graphene Electrode, Glucose, Ascorbic acid

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Cellulose Ethers, Hydrophobe Modification, Inclusion Complex., Size-exclusion Chromatography

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TRIMEB, Capillary Electrophoresis, Enantioseparation, Ionic Liquids Type Surfactants, Neutral Compounds

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DL-Lysine, Enantioselective, Kinetic Resolution, Regioselective, β-Cyclodextrin

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Amino Acid, Chiral Ligand Exchange Capillary Electrophoresis, Dipeptide, L-Hydroxyproline, γ-cyclodextrin

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Bioprocess Monitoring, Capillary Electrophoresis, Amino Acids, Laser-induced Fluorescence Detection, Beer Fermentation

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Chiral Separation, Chiral Stationary Phase, Reversed Phase-HPLC, Aryl Alcohols, Flavanoids, Adrenergic Drugs

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Conformational Enantiomers, Diazepam, HPLC on Chiral Stationary Phases, Low Temperature HPLC, Dynamic Chromatography, Enantiomerization Energy Barriers

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Chemiluminescence, Ofloxacin, Sulfonated-β-CD

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Naphthalene, Triazolophane, Mercuric Ion, Fluorescence Sensing

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Chiral Separation, Human Plasma, Propranolol, β-Blockers, β-cyclodextrin Bonded SBA-15 Chiral Stationary Phase

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Electrokinetic Chromatography, Kinetic Analysis, Methionine Sulfoxide, Sulfated β-cyclodextrin, 15-Crown-5

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Sulfonamides, Sample Preparation, Extraction, Residue Determination, Multi-class Methods, Liquid Chromatography–Tandem Mass Spectrometry

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Tel:(361) 347-6060
Fax: (361) 347-6068
e-mail: cyclolab@cyclolab.hu