Application of Matrix-Assisted Laser Desorption/Ionization Imaging Mass Spectrometry

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1. Introduction

The clarification of metabolic dynamics in lesion areas is important. Many approaches, such as high performance liquid chromatography mass spectrometry, gas chromatography mass spectrometry, immunohistochemistry, are used to define disease-related abnormalities. Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) is attracting attention as a new valuable tool. MALDI-IMS is a two-dimensional MALDI mass spectrometric technique used to visualize the spatial distribution of molecules without extraction, purification, separation, or labeling of biological samples (Cornett et al., 2007; Zaima et al., 2010b). MALDI-IMS has revealed the characteristic distribution of several biomolecules, including proteins (Caprioli et al., 1997; Groseclose et al., 2007; Morita et al., 2010), peptides (Chansela et al., 2011; Stoeckli et al., 2002), amino acids (Goto-Inoue et al., 2010b; Zaima et al., 2010a), lipids (Hayasaka et al., 2009; Murphy et al., 2009; Zaima et al., 2011a), and carbohydrates (Goto-Inoue et al., 2010b), in various tissues. The versatility of MALDI-IMS has opened a new frontier in several fields, such as pharmacology, medicine, agriculture, biology, and pathology. In this review, we describe the methodology and applications of MALDI-IMS for biological samples.

2. MALDI-MS

MALDI-MS was developed from laser desorption/ionization mass spectrometry (LDI-MS). The first LDI-MS experiment for high-mass molecules was reported in 1987 (Tanaka et al., 1987). In this experiment, a powder of cobalt metal in glycerol was used for the observation of ions with a mass to charge (m/z) ratio of 34,000. Soon afterward, MALDI-MS results of serum albumin (67,000 Da) were reported using nicotinic acid as the matrix (Karas & Hillenkamp, 1988). It was reported that MALDI-MS can detect a wide range of molecules ranging from small ($m/z < 1000$) to large molecules ($m/z > 1,000,000$) (Yates, 1998). The schema of MALDI-MS is shown in Figure 1.

In routine MALDI-MS analysis (i.e., non-imaging analysis), the analyte can be mixed with an excess of matrix. On the other hand, molecular imaging of tissue sections using MALDI-IMS requires the tissue surface to be homogeneously covered by a matrix. On-tissue

application of matrix results in the *in situ* extraction of molecules from biological tissues. The cocrystal of matrix and analyte molecules in tissue is irradiated with a pulsed laser of appropriate energy, leading to desorption and ionization of the matrix and analyte molecules. The fragmentation of analyte molecules is prevented by the incorporation of the analyte molecules into matrix crystals. The role of the optical absorption of the matrix in the transfer of energy from the laser beam to the analyte molecules is governed by Beer's law, as described previously (Karas et al., 1985). However, the mechanisms underlying the formation of charged matrix and analyte molecules in the MALDI process are not fully understood.

Fig. 1. Schema of MALDI-MS.

The matrix molecules absorb the laser energy and facilitate desorption and ionization of analyte molecules in the tissue. The homogeneous matrix cover is important for MALDI-IMS, because a heterogeneous distribution of matrix results in different ionization efficiencies of analyte molecules based on their location.

3. Methodology of MALDI-IMS

The important experimental steps for visualizing endogenous molecules or administered pharmaceutical agents in tissue using MALDI-IMS are sample preparation (such as fixation,

sectioning, and washing), choice of matrix and matrix application, measurement, and data analysis. To obtain meaningful biological images, all steps need to be carefully controlled. In this section, the basic experimental MALDI-IMS procedures are described. The schema of MALDI-IMS is presented in Figure 2.

Fig. 2. Schema of MALDI-IMS.

After biological study (a), the tissue of interest should be appropriately isolated (b). A thin section of isolated tissue is mounted on a glass slide (c), coated with matrix (d), and measured by a mass spectrometer (e). The resultant mass spectra (f) can be used for a data mining approach (g). Molecules of interest can be visualized (f) and identified by MS/MS on tissue (f).

3.1 Biological sample preparation

The samples for MALDI-IMS come from a variety of biological sources, including organs, whole animal body dosed with a pharmaceutical compound, or pathological tissues. Optimization of the sample preparation procedure according to the chemical and physical properties of analytes is important. Here, the basic sample preparation steps for MALDI-IMS are described.

3.1.1 Sample condition for MALDI-IMS

Collection and treatment procedures need to be sufficiently fast to prevent rapid tissue degradation, because the sample degradation process starts immediately after the cessation of blood flow. The most preferred sample for MALDI-IMS is a chemically unmodified fresh-frozen one. Fresh-frozen samples can be prepared using dry ice, liquid nitrogen, or liquid nitrogen-chilled isopentane, and can be preserved in a deep freezer until required. The samples should be well sealed to prevent drying during storage, and it is important to ensure that the tissue section morphology is well preserved before MALDI-IMS.

3.1.2 Fixation and embedding

Fixation of samples, such as formalin fixation, is preferably avoided because the protein crosslinking introduced by formalin fixation makes MALDI-IMS analysis difficult. However, many medical samples are routinely formaldehyde-fixed and paraffin-embedded (FFPE) just after dissection. To address this problem, the on-tissue proteolytic digestion method, in which proteins are denatured and digested by enzymes, has been developed (Djidja et al., 2009; Groseclose et al., 2007; Lemaire et al., 2007; Morita et al., 2010). The on-tissue proteolytic digestion method includes a paraffin removal step using xylene and ethanol. In the paraffin removal step, lipophilic molecules are lost; therefore, FFPE samples cannot be used for lipid imaging. When the samples are formaldehyde-fixed without paraffin-embedding, lipid imaging can be performed (Zaima et al., 2011c). However, the detected ion intensities of lipids in formaldehyde-fixed samples are lower than those in fresh-frozen ones are.

Embedding of the tissue samples in supporting material, such as an optimal cutting temperature (OCT) compound, allows the maintenance of tissue morphology and precise sample sectioning. However, supporting materials are often ionized during MALDI-MS analysis and sometimes act as ion suppressors of molecules of interest (Schwartz et al., 2003). Therefore, samples should not be embedded if precise sample sections can be prepared without embedding. When it is difficult to prepare a sample section, the use of carboxymethylcellulose (CMC) or gelatin as embedding material is recommended. Sodium CMC (2%) is reported to be used as an alternative embedding compound that does not interfere with the detection sensitivity of biomolecules in MALDI-IMS analysis (Stoeckli et al., 2006; Zaima et al., 2010a). Chen et al. reported that gelatin provides a cleaner signal background than OCT (Chen et al., 2009). Researchers should ensure compatibility between the supporting material and the biomolecules of interest.

3.1.3 Sectioning

The basic sectioning procedure for MALDI-IMS samples is same as that for pathological examination. Sections for MALDI-IMS can be prepared using a cryostat. The sample stage temperature is typically maintained between -5 and -20°C. To obtain high quality sections from tissues with high fat content (e.g., brain), or atherosclerotic lesions, breast tissue, or lipid storage disease samples lower temperatures are required. In general, 5–20- μ m-thick sections are prepared for the analysis of low-molecular-weight molecules. The use of thinner tissue sections (2–5 μ m thick) has been recommended for the analysis of high-molecular-weight molecules (range, 3–21 kDa) (Goodwin et al., 2008). Sections are usually thaw-mounted on a stainless steel conductive stage or on commercially available indium-tin oxide (ITO)-coated glass slides. We recommend the use of ITO-coated glass slides because these transparent slides enable microscopic observation of the section after MALDI-IMS. Use of adhesive film is suitable for samples for which thaw-mounted preparation of sections is challenging (e.g., bone or whole-body sections) (Stoeckli et al., 2006; Zaima et al., 2010a). The procedure for sectioning using adhesive film is shown in Figure 3. The prepared section should be immediately dried in a vacuum desiccator to avoid moisture condensation that could cause delocalization of analyte molecules in the tissue. Moisture condensation can be avoided by placing the prepared section in a dry and cold container until return to room temperature.

Fig. 3. Procedure for sectioning using adhesive film.

Attachment of adhesive film to the sample block (a). The end of the adhesive film must be anchored with tweezers to prevent adhesion of the film to the sample stage (b). After the sample section is obtained (c), the sample section on the adhesive film is attached to a glass slide (d).

3.1.4 Washing

Washing is required for peptide or protein analysis because their detection is often prevented by large amounts of easily ionized lipid species. Lipid removal simplifies mass spectra in the range of m/z 400–1000; thus, lipid removal enables the detection of low-mass peptides that are usually masked by lipid peaks. The washing method should be optimized for the target imaging molecules. Several washing protocols using organic solvents have been reported (Aerni et al., 2006; Andersson et al., 2008; Groseclose et al., 2007; Lemaire et al., 2006; Schwartz et al., 2003).

Washing is also used for removing the matrix from the tissue section after MALDI-IMS analysis. The matrix can be removed using the solvent that is used for preparing the matrix solution. For example, 2,5-dihydroxybenzoic acid (DHB) can be rapidly removed by methanol. Matrix removal enables the microscopic observation of a tissue section followed by pathological staining, such as hematoxylin and eosin (HE) staining, toluidine blue staining etc.

3.2 Matrix application

The matrix plays a central role in MALDI-MS soft ionization (Karas & Hillenkamp, 1988; Karas & Kruger, 2003). Biomolecules are softly ionized in the cocrystal with the matrix, which absorbs the laser beam energy and protects biomolecules from the disruptive energy. Protonated ion ($[M + H]^+$) or deprotonated ion ($[M - H]^-$) molecules are generally detected. Sodium adduct ion ($[M + Na]^+$) and potassium adduct ion ($[M + K]^+$) are often observed by biological sample analysis. It is very important to choose appropriate matrices for obtaining meaningful biomolecular images. An overview of the matrices used for IMS can also be found in other reviews (Chughtai & Heeren, 2010; Kaletas et al., 2009).

3.2.1 Choice of matrix

The choice of matrix used for MALDI-IMS depends on the mass range and chemical properties of the analytes. Among the many kinds of matrices, sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid [SA]) is generally used for high-molecular-weight molecules, such as proteins, while α -cyano-4-hydroxycinnamic acid (CHCA) is often used for medium-molecular-weight molecules, such as peptides. 2,6-dihydroxyacetophenone (DHA), DHB, or 9-aminoacridine (9-AA) is generally used for low-molecular-weight molecules, such as pharmaceutical compounds, lipids, or metabolites (Hattori et al., 2010; Hayasaka et al., 2009; Khatib-Shahidi et al., 2006; Sugiura et al., 2009; Woods & Jackson, 2006).

The development of new matrices is still being reported. We and other research groups recently reported the use of nanoparticles as new matrices (Hayasaka et al., 2010; McLean et al., 2005; Moritake et al., 2009; Su & Tseng, 2007; Sugiura & Setou, 2010). For example, iron oxide nanoparticles enable the visualization of sulfatide and phospholipid distribution (Ageta et al., 2009; Taira et al., 2008), silver nanoparticles can be used for the analysis of fatty acids (Hayasaka et al., 2010), and gold nanoparticles are appropriate for the sensitive detection of glycosphingolipids, such as sulfatides and gangliosides (Goto-Inoue et al., 2010a).

3.2.2 Matrix application

There are various methods for applying the matrix onto the section, such as deposition, spraying, and sublimation. The matrix application method also influences analyte extraction efficiency. Compared to other methods, the deposition of matrix solution using automatic depositing robotic devices, such as a chemical inkjet printer (ChIP-1000; Shimadzu Corporation, Kyoto, Japan), increases signal sensitivity, but decreases spatial resolution (Aerni et al., 2006; Chansela et al., 2011; Morita et al., 2010). The other limitation of the inkjet printer is capillary clogging, which occurs when highly concentrated matrix solutions are used. Spraying is the most frequently used method in MALDI-IMS. Using this method, an entire tissue section can be homogeneously coated with relatively small crystals in a short time without special equipment. For its operation, several instruments, including Thin layer chromatography (TLC) sprayers and artistic airbrushes, are available; we use a metal airbrush with a 0.2-mm nozzle because of its simple and easy-to-handle design. This method requires skillful operation because some airbrush parameters are hand-operated. If there is an excess of matrix solution on the tissue, an inhomogeneous crystal can be formed with analytes that have migrated from their original location; on the other hand, if not enough matrix solution is sprayed and it evaporates without sufficiently moisturizing the tissue section, analytes cannot

be adequately extracted from the tissue section. The operation should be performed at a constant room temperature and humidity. Beginners are recommended to practice spraying until homogeneous matrix spraying can be reproducibly achieved. Sublimation is a new method for applying matrix to tissue sections (Hankin et al., 2007). Using this technique, a matrix can be applied uniformly over a large sample plate in a few minutes without solvents. Additionally, previous reports demonstrated that this method increases analyte signal and that the fine microcrystals formed from the condensed vapor reduce the image resolution limitation caused by crystal size (Dekker et al., 2009; Vrkoslav et al., 2010).

3.3 Measurement and data analysis

3.3.1 Measurement

MALDI-IMS should be performed as soon as possible after matrix application, regardless of the coating method. The procedure to obtain a good spectrum in MALDI-IMS is almost the same as that for traditional MALDI-MS; mass range, detector gain, and laser power must be optimized. From the mechanical setting perspective, there are 3 differences between MALDI-MS and MALDI-IMS. The first difference is the above-mentioned matrix application. The second difference is the need for focusing of the laser beam. To obtain meaningful biological images by MALDI-IMS, the laser spot size should be reduced to 10–50 μm . The third difference is that a two-dimensional region must be set for analyses. The scan pitch, which signifies the distance between laser irradiation spots, must be fixed. The limitation of the scan pitch, which decides the spatial resolution of the image, depends on the laser spot size and mechanical movement control of the mass spectrometer sample stage. We have developed a new instrument (Mass Microscope) that can move the sample stage by 1 μm , and in which the finest size of the laser diameter is approximately 10 μm (Harada et al., 2009). The measurement time depends on the number of data spots, the frequency of the laser, the number of shots per spot, and the time required to move the sample stage. For example, when researchers select the region of interest as a $1 \times 1 \text{ mm}^2$ area with a 10- μm scan pitch (10,000 data points), it takes about 1 h to complete the measurement using a mass microscope equipped with a 1000-Hz laser (100 shots/data point).

MALDI-IMS ionizes numerous compounds in a tissue at the same time. Sometimes, we detect multiple molecules with the same m/z value. In such cases, a new imaging technique, “MS/MS imaging,” is effective. Using this technique, we can separate each ion derived from their specific fragment ions. Some reports have described the use of MS/MS imaging for IMS of endogenous metabolites and an exogenous drug (Khatib-Shahidi et al., 2006; Porta et al., 2011). Additionally, the combination of ion-mobility separation with MALDI-IMS provides a unique separation dimension to further enhance the capabilities of IMS (Jackson et al., 2007; McLean et al., 2007; Stauber et al., 2010). It can be used to produce images without interference from background ions of similar mass, and this can remove ambiguity from imaging experiments and lead to a more precise localization of the compound of interest.

3.3.2 Data analysis

A large amount of data (a few gigabytes) is obtained from MALDI-IMS; therefore, visualization software packages that can rapidly and efficiently analyze enormous spectra have been developed. BioMap (a free software; Novartis, Basel, Switzerland), FlexImaging

(Bruker Daltonics, Bremen, Germany), and ImageQuest (Thermo Fisher Scientific, CA, USA) are generally used for visualization. For biomarker analysis of the MALDI-IMS dataset, data mining should be used (Hayasaka et al., 2011; Zaima et al., 2011b; Zhang et al., 2004). Data mining software effectively reduce the number of biomarker candidates (Hayasaka et al., 2011; Zhang et al., 2004). We previously reported the use of principal component analysis (PCA) to discover different biomolecules in starvation-induced fatty livers and normal livers (Zaima et al., 2009). Hierarchical clustering was also used to analyze the data obtained from gastric cancer and non-neoplastic mucosa tissue sections (Deininger et al., 2008). Several studies have reported the discovery of biomarkers using MALDI-IMS (Bakry et al., 2011; Ducret et al., 2006; Hong & Zhang, 2011; Solassol et al., 2009; Zaima et al., 2011b).

4. Instruments

The requirement for performing IMS is the availability of an *x*-axis-*y*-axis moving stage with electronic controls. Most modern MS instruments produced by major MS hardware companies (*i.e.*, Shimadzu, ThermoFisher Scientific, Bruker Daltonics, Applied Biosystems, Waters) can be adapted for MALDI-IMS. Time of flight (TOF) is the most widely used technology. TOF analyzers allow the separation of ionized accelerated molecules according to their *m/z* ratio. TOF-MS offers suitable performance for MALDI-IMS, namely, good transmission ratio (50–100%), sensitivity, mass range, and repetition rate. However, TOF-MS lacks the capability to perform effective tandem MS experiments. This disadvantage of TOF-MS was overcome with the introduction of hybrid analyzers, such as a combination of quadrupole mass analyzer and TOF (so-called qTOF), combination of quadrupole ion trap (QIT) and TOF (so-called QIT-TOF), combination of ion mobility spectrometry (IMS) and TOF (so-called IMS-TOF), or a combination of two TOF mass spectrometers (so-called TOF-TOF). These combination systems revolutionized the application of TOF-MS systems for structural analysis with tandem MS. In general, the first system is used to select a precursor ion for fragmentation, while the second TOF system is employed for fragment analysis. Other mass analyzers (and their combinations), such as linear ion trap (LIT) (Landgraf et al., 2009; Wiseman et al., 2006; Zaima et al., 2010a), triple quadrupole (QqQ) (Hopfgartner et al., 2009; Porta et al., 2011), and Fourier transform ion cyclotron resonance (FTICR) (Taban et al., 2007), are used for MALDI-IMS. The advantages of commercially available LIT instruments are miniaturization, capability of sample analysis on nonconductive glass slides, MALDI performance at intermediate pressure, and superior performance on multistage MS. The QqQ system allows quantitative analysis and single or multiple reaction monitoring (SRM/MRM). The FTICR system offers very high mass resolving power and high mass measurement accuracy.

5. Applications of MALDI-IMS

5.1 Imaging mass spectrometry-based histopathologic examination

Recently, we applied MALDI-IMS for pathologic examination of atherosclerotic aorta (Fig. 4). We named it imaging mass spectrometry-based histopathologic examination (IbHE) (Zaima et al., 2011c). IbHE revealed the characteristic distribution of biomolecules in smooth muscle cells, lipid-rich regions, and calcified regions of an atherosclerotic lesion obtained from aortic roots of apolipoprotein E (ApoE)-deficient mice. We found that phosphatidylcholine (PC), which contains arachidonic acid (20:4) (*m/z* 804.5), was distributed in the smooth muscle cells of the atherosclerotic lesion. Cholesterol linoleate (CE

18:2) (m/z 671.6) and cholesterol oleate (CE 18:1) were characteristically distributed in lipid-rich regions, and the ion at m/z 566.9 was localized in the calcified region. These biomolecules were hardly detected in the normal aortic roots of ApoE-deficient mice. We applied this method to other vascular diseases, such as varicose veins, arteriovenous fistulae, abdominal aortic aneurysm, and triglyceride deposit cardiomyovasculopathy, and observed the characteristic distribution of biomolecules (Tanaka et al., 2010; Tanaka et al., 2011). In the analysis of several vascular diseases with atherosclerotic lesions, we often observed ectopic TG distribution. Although the role of TG in the evolution of atherosclerosis remains unknown, there is a possibility that TG plays an important role in the evolution of some kinds of atherosclerosis, as we previously found that characteristic atherosclerosis accumulated TG in aortic lesions, while the accumulated cholesterol was normal (Hirano et al., 2008). The reexamination of vascular diseases by IbHE may result in new findings, because IbHE can visualize the localization of low-molecular-weight molecules, which are rarely visualized by other techniques. We believe IbHE is of considerable value as a new histopathological examination because IbHE can visualize metabolic abnormalities in disease.

Fig. 4. Representative molecular images of specific ions in a mouse atherosclerotic lesion.

Visualization of biomolecules in atherosclerotic roots (a-j). Scale bar, 100 μ m. Specific ion images of region 1 (a and b) and the combined image of m/z 671.6 and 673.6 (c). Specific ion images of region 2 (d and e) and the combined image of m/z 804.5 and 832.5 (f). Specific ion images of region 3 (g) and the monochrome image of m/z 566.9 (h). Comparison of HE staining (i) and the merge images of regions 1, 2, and 3 (j). An image of non-atherosclerotic aortic roots of mice at 12 weeks of age (k-m). Scale bar, 200 μ m. HE staining after IMS (k). Oil red O staining (l). Immunostaining of α -actin, which is a marker for smooth muscle cells (m). Merge image of CE (18:2) and CE (18:1) (n). Merge image of PC (diacyl 16:0/20:4) and PC (diacyl 18:0/20:4) (o). Ion image of m/z 566.9 (p). "Reprinted from *Atherosclerosis*, 217, 2, Zaima et al., Imaging mass spectrometry-based histopathologic examination of atherosclerotic lesions, 430., Copyright (2011), with permission from Elsevier."

5.2 IMS for exogenous drugs

MALDI-IMS is a powerful tool for visualizing the distribution of exogenous drugs and their metabolites. Porta et al. reported the visualization of the distribution of cocaine and its metabolites down to a concentration of 5 ng/mg in intact single hair samples from chronic users (Porta et al., 2011) (Fig. 5).

Fig. 5. Imaging of cocaine in hair samples H7 and H5. (H7 and H5 are sample names used in this article)

Optical image of hair sample H7 (a). MALDI-SRM/MS image based on the SRM trace of COC (m/z 305 > m/z 182) for five replicates of single hair samples from individual H7 (b) and single hair analysis from individual H5 (c). The quantitative results from LC-SRM/MS routine analysis were as follows: 130 ng/mg (H7, whole sample); 4.9 ng/mg (H5, segment 0–10 mm), and 8.5 ng/mg (H5, segment 10–50 mm). SRM; selected reaction monitoring. “Reprinted with permission from Porta et al., 2011. Copyright 2011 American Chemical Society.”

MALDI-IMS is also applicable to pharmacokinetic analysis. As a Food and Drug Administration (FDA)-mandated pharmacokinetic test, whole-body autoradiography (WBA) is widely performed to determine spatial and quantitative information about a drug compound. Although much information can be acquired by WBA, it has several limitations. First, WBA requires the compound of interest to be radioactively labeled. Furthermore, the detected signal does not distinguish between the original radiolabeled compound and its metabolites that have retained the radiolabel. To complement the disadvantage of WBA, MALDI-IMS and WBA have recently been used together. The combination of MALDI-IMS and

WBA makes it possible to obtain more reliable data for absorption, distribution, metabolism, and excretion of drugs (Atkinson et al., 2007; Caprioli et al., 2008; Clench et al., 2008; Stoeckli et al., 2006). The application of MALDI-IMS to pharmacokinetics in a whole-body mouse section was first reported by Rohner et al. in 2005 (Rohner et al., 2005). In this study, they showed a good correlation between WBA and MALDI-IMS data. Figure 5 shows the simultaneous visualization of drug and metabolites in a whole-rat sagittal tissue section (Khatib-Shahidi et al., 2006). Khatib-Shahidi et al. visualized the temporal distribution of dosed olanzapine (brand name Zyprexa) (8 mg/kg) and its metabolites. In this study, MALDI-IMS was further extended to detect proteins from organs present in a whole-body section.

Fig. 6. Detection of drug and metabolite distribution at 6 h post-dose in a whole-rat sagittal tissue section by a single IMS analysis.

Optical images of a 6 h post-olanzapine (OLZ)-dosed rat tissue section across 4 gold MALDI target plates (A). Organs are outlined in red. MS/MS ion image of OLZ (m/z 256) (B). MS/MS ion image of N-desmethyl metabolite (m/z 256) (C). MS/MS ion image of 2-hydroxymethyl metabolite (m/z 272) (D). Scale bar, 1 cm. "Reprinted with permission from Khatib-Shahidi et al., 2006. Copyright 2006 American Chemical Society."

6. Conclusions

MALDI-IMS can be applied to pathological examinations leading to the discovery of potential targets for new drugs, and for the distributional analysis of exogenous drugs in animal and human tissues. We recently used MALDI-IMS in the discovery of metabolites that have pharmacological effects on natural resources. MALDI-IMS will become an essential tool for molecular imaging in pharmacology in the near future.

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8. References

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The history of pharmacology travels together to history of scientific method and the latest frontiers of pharmacology open a new world in the search of drugs. New technologies and continuing progress in the field of pharmacology has also changed radically the way of designing a new drug. In fact, modern drug discovery is based on deep knowledge of the disease and of both cellular and molecular mechanisms involved in its development. The purpose of this book was to give a new idea from the beginning of the pharmacology, starting from pharmacodynamic and reaching the new field of pharmacogenetic and ethnopharmacology.

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