Improved Skin Delivery of Voriconazole with a Nanostructured Lipid Carrier-Based Hydrogel Formulation

Seh Hyon Song, Kyung Min Lee, Jong Boo Kang, Sang Gon Lee, Myung Joo Kang, and Young Wook Choi

Abstract

In order to develop topical preparations of voriconazole (VRC) for the treatment of mycotic infections of the skin, a nanostructured lipid carrier-based hydrogel (NLC-gel) formulation was developed and its physical characteristics, in vitro skin permeation, and retention profiles were examined. A VRC-loaded NLC dispersion, consisting of Precirol ATO 5, Labrafir 1944 CS, and Tween 80, was prepared by high-pressure homogenization and embedded into Carbopol 940 hydrogel. The lipid nanoparticles in the hydrogel were approximately 210 nm in size, with a spherical shape and zeta potential of −30 mV. In a skin permeation study using a Franz diffusion cell mounted with depilated mouse skin, the NLC-gel was superior to conventional cream and microemulsion-based gel formulations, showing 2.8- and 1.7-fold greater flux values, respectively. In addition, the NLC-gel led to markedly greater accumulation of VRC in deeper skin layers as compared with the reference formulations. In conclusion, the novel topical formulation reported here represents an alternative treatment for skin infections such as candidiasis, with less potential for systemic adverse effects than oral therapy.

Key words voriconazole; nanostructured lipid carrier; hydrogel; topical delivery

Voriconazole (VRC), a second-generation triazole derived from fluconazole, is a broad-spectrum anti-fungal agent that inhibits cytochrome P450-dependent 14α-lanosterol demethylation, which is a vital step in cell membrane ergosterol synthesis. VRC is active against all Candida species that have acquired resistance to fluconazole and is currently used for the treatment of aspergillosis and candidiasis infections in the abdomen, kidney, bladder wall, wounds, and skin. Despite its advantageous pharmacological activity, systemic exposure to VRC as a result of oral and/or intravenous administration can cause several side effects, not fatal but considerable, including photopsia, abdominal pain, and visual hallucinations. In addition, VRC triggers elevations in hepatic enzyme levels in patients at a high risk of pharmacokinetic drug–drug interactions.

A need exists for a topical delivery system for VRC to overcome limitations in oral and intravenous treatment, and which will be particularly useful against candidiasis in wounds and skin tissue. Topical drug application provides higher local tissue levels, more rapid drug delivery, and lower systemic exposure than oral administration. However, because of the poor aqueous solubility of VRC, solubilization of the drug is necessary for delivery in a topical formulation. Furthermore, a penetration-enhancing system is required to achieve therapeutic concentrations in stratum corneum and deeper skin layers, including epidermis and/or dermis.

In recent years, nanostructured lipid carriers (NLCs) have emerged as a promising topical delivery system for pharmaceutical and cosmetic molecules, especially for the delivery of lipophilic compounds. NLC dispersions composed of a solid lipid matrix with a liquid–lipid are colloidal nanoparticles in the submicron range of approximately 40 to 1000 nm. Compared with conventional topical delivery systems, such as creams, tinctures, and emulsions, solid lipid-based particulate systems have several desirable features such as low toxicity of constituents, the ability to protect the incorporated drug from degradation by immobilization in the solid particle matrix, and the ability to release loaded molecules in a controlled manner. In particular, after topical administration lipid particles can closely contact the stratum corneum due to their small size and increase drug penetration into the skin, with improved skin hydration capacity.

The purpose of this study is to formulate an NLC dispersion-based hydrogel (NLC-gel) with VRC for facilitated drug delivery into the skin, including the stratum corneum and epidermis. A VRC-loaded lipid carrier system was successfully prepared using a high-pressure homogenization technique and embedded into a gel base for topical application. The NLC dispersion was characterized with respect to particle size, surface charge, drug entrapment efficiency and amount, and morphological features. In vitro skin permeation and retention profiles of VRC in the delivery-enhancing NLC-gel formulation were compared to conventional oil-in-water cream (CC) and microemulsion-based gel (ME-gel) preparations.

Experimental

Materials VRC (purity >98%) was purchased from Dr. Reddy’s Laboratories Ltd. (Hyderabad, India). Precirol® ATO 5 (glycerol distearate, melting point: 56°C) and Labrafir M 1944 CS (oleoyl macrogol-6 glycerides) were kindly gifted by Gattefosse (Nanterre, France). Tween® 80 (polysorbate 80) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). Carbopol 940 NF polymer was purchased from Lubrizol Advanced Materials, Inc. (Cleveland, OH, U.S.A.). Carnosine (β-alanyl-L-histidine) was purchased from Tokyo Chemical Industry Co. (Toshiba, Japan). All other chemicals and reagents were purchased from commercial sources and were of analytical grade. Doubly distilled water was used for all experiments.

The authors declare no conflict of interest.

# These authors contributed equally to this work.
**Animals** Male 5-week-old ICR mice were purchased from Orient Bio (Kyungki-Do, Korea). Mice were kept in specific pathogen-free conditions with food and water freely available. All animal experiments were performed in accordance with the NIH Principles of laboratory animal care guidelines (NIH publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Chung-Ang University in Seoul, Korea.

**HPLC Assay of VRC** Quantitative determination of VRC was performed by HPLC. The system consisted of separation modules (Waters 2695), UV detector (Waters 2489), and a data station with Empower3 software, which were obtained from Waters Corporation (Milford, MA, U.S.A.). VRC was separated using an L1 column (4.6mm×25cm; 5-mm packing) with acetonitrile:7.8g/L sodium dihydrogen phosphate buffer (48:52) as a mobile phase, at a flow rate of 0.7mL/min. The injection volume was 50µL and VRC was detected at 254 nm. The intraday and interday precision and accuracy were based on six different standard concentrations. Calibration curves were used to determine the limit of detection and limit of quantitation.

**Preparation of NLC Dispersion** An NLC aqueous dispersion was prepared using a high-pressure homogenization technique as described previously. Briefly, VRC (0.25% w/v) was dissolved in a mixture of Precirol® ATO 5 (solid lipid, 5.6%) and Labrafil M 1944 CS (oil, 2.4%) at 85°C. Tween 80 (stabilizer, 1.5%) was dissolved in distilled water at 85°C for injection. The aqueous phase was added to the oil phase and sonicated (Model 2210, Branson Ultrasonics Co., Danbury, CT, U.S.A.) for 5min. The resulting pre-emulsion was passed through a microfluidizer (Microfluidizer M-110S, Microfluidics, Newton, MA, U.S.A.) for 15 cycles at 500 bar. Next, the NLC dispersion was cooled and stored at room temperature.

**Preparation of NLC-Gel** NLC-embedded hydrogel was prepared to increase viscosity and improve skin applicability. Carbopol 934P (0.6%) served as a gelling agent and was dispersed in the NLC aqueous dispersion, which contained 8% lipid ingredients, using a stirrer at a speed of 300rpm for 5min. Next, the dispersion was homogenized at 11,000rpm for 2min. Finally, the dispersion was neutralized by addition of 0.6% (w/v) carnosine to adjust the pH of the gel formulation to within the range of 5.5 to 6.5.

**Preparation of CC Formulation** A conventional oil-in-water type cream containing 0.25% (w/w) VRC was prepared as follows. VRC was dissolved in an oil phase consisting of mineral oil (7%), stearic acid (5%), cetyl alcohol (2%), and polyglyceryl-3-methylglucose distearate (3%). The oil phase was added to an aqueous phase consisting of glycerin (10%) and distilled water, homogenized for 10min, degassed, and cooled under vacuum. Cream formulations were stored for approximately 24h before undergoing evaluation.

**Preparation of ME and ME-Gel** An ME aqueous dispersion containing 0.25% of VRC was prepared by dissolving the drug in an oil phase consisting of Labrafil 1944 CS, Tween80, and Transcutol, in a weight ratio of 23:58:19. After dissolving VRC, the oil phase was added to distilled water and stirred for 10min. An ME-gel was prepared from the ME for use as a reference using the same preparation method as that used for the NLC-based gel.

**Physical Characterization of NLC and NLC-Gel** Particle size and zeta potential: Samples were diluted 1:10000 with distilled water, and particle size distribution and zeta potential were assayed using a dynamic light-scattering particle size analyzer (Zetasizer Nano-ZS, Malvern Instrument, Worcestershire, U.K.), with a 50mW laser at a scattering angle of 90°. All measurements were carried out in triplicate under ambient conditions.

Drug encapsulation efficiency and loading amount: The drug encapsulation efficiency (%) of VRC in the NLC system was determined by an ultrafiltration centrifugation method. The amount of unencapsulated drug in the water phase was determined indirectly by ultrafiltration using centrifugal filter tubes (Amicon Ultra-4; EMD Merck Millipore, Billerica, MA, U.S.A.) with a molecular weight cut-off of 30kDa. The amount of the anti-fungal agent encapsulated was calculated as the difference between the total amount used to prepare the NLC and the amount of VRC that remained in the aqueous phase after isolation of the system. The concentrations of VRC were measured using HPLC, and the efficiency (%) was calculated using the following formula: \( \frac{|D−C|×100}{D} \), where \( D \) is the total amount of drug added and \( C \) is the amount of drug detected in the water phase. To determine the amount of VRC loaded into the lipid carrier, 100µL of NLC dispersion was diluted with methanol and heated in a water bath at 70°C for 30min to dissolve the VRC from the lipid carriers. Drug loading (mg VRC/g lipid) was calculated using the following formula: \( \frac{R−C}{L} \), where \( R \) is the total amount of the active agent remaining in the NLC dispersion, \( C \) is the amount of drug detected in the water phase, and \( L \) is the total amount of lipid initially added. All measurements were carried out in triplicate under ambient conditions.

**Morphological Features of NLC and NLC-Gel** The morphological features of NLC and NLC-gel were observed using a scanning electron microscope (Sigma, Carl Zeiss). Prior to photography, the samples were coated with a thin layer of gold using a sputter coater unit (Structure Probe Inc., West Chester, PA, U.S.A.). Scanning electron microscope (SEM) photographs were taken using a scanning electron microscope operated at an acceleration voltage of 15kV.

**In Vitro Drug Release Study** The release of VRC from NLC and NLC-gel was evaluated using a Franz diffusion cell mounted with a dialysis membrane (Spectra/Per dysis membrane with 8kDa MWCO, Spectrum Labs, Rancho Dominguez, CA, U.S.A.). The receptor phase was filled with phosphate buffered saline (PBS, pH 7.4) as an acceptor medium and water-jacketed at 32°C to mimic human skin. The dispersion, containing 2.5mg of VRC, was applied to the donor compartment, which had an available diffusion area of 1.76cm². Aliquots (300µL) were withdrawn from the receptor compartment (11mL) at predetermined time intervals and immediately replaced with an equal volume of PBS solution.

**In Vitro Skin Permeation and Retention Study** An in vitro permeation study was conducted with vertical Franz diffusion cells as described previously. Skin tissue was obtained from ICR mice (5 weeks old, 26±1 g), from whom the dorsal hair was carefully removed using electric clippers, and the skin was rinsed with phosphate buffer. The receptor compartment was filled with 10mPBS (pH 7.4, 11mL) solution and was maintained at 32°C. A formulation containing
VRC (1.25 mg) was applied to the skin surface, which had an available diffusion area of 1.76 cm². Aliquots (0.5 mL) in the receptor phase were withdrawn at predetermined time intervals and analyzed by HPLC. The cumulative amount of drug permeated per unit area was plotted as a function of time.

At the end of the skin permeation study (12 h), the skin tissue was thoroughly washed 5 times with PBS. VRC retained in the stratum corneum layer was measured by the tape-stripping method using cellophane tape (Scotch Brand, 3M, Saint Paul, MN, U.S.A.). Next, the remaining skin layer was cut into small pieces. Tissue was homogenized with diluent for 5 min, and after centrifuging at 12000 × g for 10 min, the VRC content in the supernatant was measured by HPLC.

Statistical Analysis All data were expressed as mean ± standard deviation (S.D.). Statistical significance was determined using Student’s t-test and was considered to be significant at p<0.05, unless otherwise indicated.

Results and Discussion

VRC Assay Validation The calibration curve was linear in the VRC concentration range of 5–100 µg/mL (r²=3622.1x+4771, r²=0.9999). The calibration standards for intraday and interday accuracy and precision are listed in Table 1. Intraday and interday precision ranged from 0.24 to 0.82% and from 2.57 to 3.31%, respectively. The maximum values for intraday and interday variation at 5 µg/mL were 0.71 and 1.82, respectively.

Table 1. Intra- and Inter-Day Accuracy and Precision for VRC by HPLC Assay

<table>
<thead>
<tr>
<th>Spiked concentration (µg/mL)</th>
<th>Detected concentration (µg/mL)</th>
<th>Precision (R.S.D)%</th>
<th>Accuracy (% DEV)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day variation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.96±0.04</td>
<td>0.82</td>
<td>0.71</td>
</tr>
<tr>
<td>20</td>
<td>19.93±0.05</td>
<td>0.24</td>
<td>0.37</td>
</tr>
<tr>
<td>60</td>
<td>60.21±0.29</td>
<td>0.48</td>
<td>−0.35</td>
</tr>
<tr>
<td>Inter-day variation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.91±0.15</td>
<td>3.05</td>
<td>1.82</td>
</tr>
<tr>
<td>20</td>
<td>19.88±0.66</td>
<td>3.31</td>
<td>0.58</td>
</tr>
<tr>
<td>60</td>
<td>60.45±1.55</td>
<td>2.57</td>
<td>−0.75</td>
</tr>
</tbody>
</table>

a) Data are expressed as mean±S.D. b) Relative standard deviation=(S.D./mean)×100. c) Percentage deviation=(spiked concentration−measured concentration)/spiked concentration×100.

Preparation and Physical Characteristics of NLC and NLC-Gel To formulate an NLC dispersion system for a poorly water-soluble drug like VRC, the selection of a suitable solid lipid, liquid lipid, and surfactant is critical. The drug-loading capacity of the NLC predominantly depends on the solubility of the drug in the lipid ingredients. To select a suitable lipid for the VRC-loaded NLC, solid lipids in the generally recognized as safe (GRAS) category were screened for suitable lipid nanoparticle colloidal stability (data not shown). The droplet size of the ME dispersion approximated 193 nm, which is appropriate for skin delivery. When the particle size is less than 200 nm, the lipid nanoparticles formed a dense monolayer on the skin and retarded the evaporation of water from the skin, which facilitated the drug penetration into deeper layers of skin by forming a transcutaneous hydration gradient. The low polydispersity index (PDI) of less than 0.3 indicates a narrow and homogeneous size distribution. Surface charges in the NLC particles were measured as approximately −30 mV, which was sufficient to prevent aggregation of lipid nanoparticles via electric repulsion. Since drug molecule basically did not influence the zeta potential (data not shown) and Polysorbate 80 is a non-ionic surfactant, the same ionization and/or hydrolysis of fatty ester groups in Labrafil M1944CS and/or Precirol ATO 5 was possibly responsible for the negative surface charge. According to the literature, when the absolute value of zeta potential for colloidal system is higher than 30 mV, the particles are electrochemically stable under the storage condition because the surface charge prevents aggregation of the particles. The NLC aqueous dispersion system showed a high loading efficiency greater than 86%, which indicated that VRC was efficiently incorporated into the lipid particles with their lipophilicity (Log P value of 1.8). The NLC in the hydrogel was also physically characterized, and the particle size of the lipid nanoparticles was measured at 212 nm, which was nearly equivalent to that of the NLC aqueous dispersion. The PDI value less than 0.3 indicated a narrow and homogeneous distribution in the matrix, without particle aggregation or destruction.

A CC prepared as a reference appeared as a white, opaque, and homogenous semifluid with no bleaching or phase separation. The drug content in the formulations was greater than 97%. The ME was formulated based on our preliminary study, including construction of a ternary phase diagram, measurement of droplet size and its homogeneity, and determination of colloidal stability (data not shown). The droplet size of the ME and ME-gel was identical and in the range of 60–100 nm with a narrow size distribution (PDI <0.3).

Morphological Features of NLC and NLC-Gel Figure 1 shows SEM images of an NLC aqueous dispersion (Figs. 1A, B) and NLC-gel (Figs. 1C, D). Micrographs of NLC dispersions showed spherical nanoparticles in the nanometric range, in agreement with dynamic light scattering analyses.

Table 2. Characteristics of VRC-Loaded NLC in Aqueous Dispersion and Gel Formulation

<table>
<thead>
<tr>
<th></th>
<th>NLC dispersion</th>
<th>NLC-gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Average size (nm)</td>
<td>193.5±3.4</td>
<td>212.2±20.9</td>
</tr>
<tr>
<td>Polydispersity index</td>
<td>0.272±0.010</td>
<td>0.288±0.030</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>−27.8±0.8</td>
<td>−26.5±1.2</td>
</tr>
<tr>
<td>Entrapment efficiency (%)</td>
<td>86.2±1.7</td>
<td>N.D.a</td>
</tr>
<tr>
<td>Loading amount (mg VRC/g lipid)</td>
<td>23.3±1.0</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

a) Not determined. Data are expressed as mean±S.D. (n=3).
The particles were observed to be slightly aggregated in the SEM image (Fig. 1A), which was probably a result of drying during sample preparation prior to analysis. Morphological features of lipid nanoparticles embedded in the gel base were also observed (Figs. 1C, D). Lipid nanoparticles were consistently dispersed in the gel base, and retained their spherical shape with a narrow size distribution. From these findings, we concluded that the NLC incorporated into the hydrogel homogeneously, while preserving the advantageous properties of NLC dispersion, such as small particle size and homogeneity.

**In Vitro Drug Release Study** In vitro release of VRC from the NLC-based formulations was evaluated using a Franz diffusion cell, where the sink conditions were maintained. The solubility of VRC in PBS solution was determined as approximately 0.65 mg/mL. This equilibrium solubility guarantees the sink conditions for VRC, because the total amount of drug applied to the donor phase was 2.5 mg and the volume of the receptor phase was 11 mL in the Franz diffusion cell.

As expected, the release rate of VRC became retarded when the VRC-loaded lipid nanoparticles were incorporated into the gel base. The NLC dispersion showed a 95% release of VRC, whereas the NLC-gel released up to 76% of VRC within 24 h (Fig. 2). The release profiles indicated that embedding NLC into hydrogel effectively controlled the release of VRC, because the release of VRC from the NLC-gel was a combination of the release of the drug from lipid carriers and subsequent diffusion through the micro-channel structures of the carbopol gel. In a clinical setting, this sustained release by hydrogel would provide the drug over a prolonged period of time.

**In Vitro Skin Permeation and Retention Study** The in vitro permeation profiles of VRC in NLC and NLC-gel in mouse skin are shown in Fig. 3. Topical formulations including CC, ME and ME-gel containing 0.25% VRC were tested as controls. The skin permeation and retention studies were performed for 12 h to correspond to clinical application time. VRC showed rapid penetration through the skin with no lag time after application, indicating that steady-state permeation was reached quickly. The slope of the NLC-based formulations NLC and NLC-gel were much steeper than those of the other preparations. The relative magnitudes of VRC penetration at 12 h was as follows: NLC>NLC-gel>ME>ME-gel>CC. The permeation parameters calculated from slopes of permeation profiles are listed in Table 3. NLC aqueous dispersion remarkably increased the amount of VRC that had permeated at 12 h: 1.9-fold versus ME, and 4.0-fold versus CC. In the case of NLC-gel, although the flux value decreased by 70% compared to NLC dispersion, the novel topical preparation had a high permeation rate and amount of VRC permeated, along with better topical application. The NLC-gel showed a greater amount of VRC permeated at 12 h than ME-gel or CC, with a 1.7-fold and 2.8-fold greater permeation, respectively.

Formulation effects on skin permeation of VRC were further categorized into 2 variables: NLC formulation and hydrogel incorporation (Fig. 4). The enhancement ratio (ER) was expressed as a relative ratio of the flux values. NLC and NLC-gel exhibited ER of 1.8 and 1.6 versus ME and ME-gel, respectively, clearly demonstrating that NLC and NLC-gel were superior to ME and ME-gel formulations for VRC skin...
In addition, Fang et al. reported that coenzyme Q10-loaded NLC provided a greater amount of drug in the receptor medium than an ME-based formulation. 

Moreover, combination with hydrogel slightly decreased the flux values in both NLC and ME formulations. NLC-gel and ME-gel exhibited ER values of 0.70 and 0.79 versus those of NLC and ME aqueous dispersions, respectively. Because the incorporation of lipid particulates into the hydrogel matrix retarded the release of VRC, as shown in the release study. Nevertheless, NLC-gel showed a higher flux value than ME-gel and ME solution (p<0.05).

Finally, the amount of VRC (µg/mg) retained in distinct skin layers after the 12h permeation study was investigated (Fig. 5). The amount of VRC deposited in the stratum corneum was similar across all preparations, and ranged from 0.25 to 0.32 µg/mg. This result suggests that VRC was readily bound, and accumulated in the keratinized stratum corneum layer due to its high lipophilicity, with little influence from formulation variables. In contrast, in deeper layers the amount of drug deposited (µg/mg) using NLC-gel was remarkably high compared to that of ME, ME-gel and CC formulations. The amount of drug in the epidermis and/or dermis after NLC-gel treatment was 2.0-, 2.1- and 3.7-fold greater than ME, ME-gel and CC, respectively. The relative magnitudes of the total amount of VRC retained in the whole skin tissue among the formulations were as follows: NLC and NLC-gel>ME>ME-gel>CC. This result was similar to the order of formulations from the skin permeation assay. It appears that lipid nanoparticles could closely contact the junctions of corneocyte clusters and furrows, possibly favoring drug accumulation, regardless of gelation. In addition, the high binding affinity of NLC lipids to lipophilic skin layers and other tissues contributed to skin accumulation of VRC after topical application of both NLC and NLC gel. From these findings, we concluded that the novel topical formulation used in this study led to increased skin delivery of VRC.

Table 3. Skin Permeation Parameters of VRC through Depilated ICR Mouse Back Skin in Various Formulations

<table>
<thead>
<tr>
<th></th>
<th>NLC</th>
<th>NLC-gel</th>
<th>ME</th>
<th>ME-gel</th>
<th>CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flux (µg/mg/h/cm²)</td>
<td>25.5±2.4</td>
<td>17.7±3.3</td>
<td>13.8±5.7</td>
<td>10.9±5.0</td>
<td>6.4±1.2</td>
</tr>
<tr>
<td>Permeated (%)</td>
<td>43.3±2.8</td>
<td>30.1±3.3</td>
<td>22.5±5.3</td>
<td>18.1±4.7</td>
<td>10.9±1.3</td>
</tr>
<tr>
<td>ER (c)</td>
<td>4.0</td>
<td>2.8</td>
<td>2.2</td>
<td>1.7</td>
<td>1.0</td>
</tr>
<tr>
<td>ER (b)</td>
<td>25.5</td>
<td>22.5</td>
<td>18.1</td>
<td>10.9</td>
<td></td>
</tr>
</tbody>
</table>

a) Flux was obtained from the slope of the permeation profile in Fig. 3. b) Calculated from the cumulative amount permeated at 12 h divided by the concentration of VRC loaded into the samples. c) Enhancement ratio calculated by relative ratio to CC. Data are expressed as mean±S.D. (n=5).

Conclusion

A VRC-loaded NLC dispersion consisting of Precirol ATO 5, Labrafil 1944 CS, and Tween 80 was successfully prepared by high-pressure homogenization, and subsequently embed-
ded into Carbopol 940 hydrogel. Incorporation of VRC-loaded NLC into the hydrogel effectively regulated drug release from the formulation, while preserving advantageous physical properties of the NLC dispersion, including small particle size and homogeneity. In a skin permeation and retention study, the NLC-embedded gel formula exhibited significantly higher skin permeation and retention of VRC than CC and micro-emulsion-based formulations, and thus maintained the skin delivery-enhancing properties of NLC. Therefore, the novel NLC-gel system described herein represents a promising tool for topical delivery of VRC, a poorly soluble triazole molecule with a broad anti-fungal spectrum.

Acknowledgment This study was supported by Seoul R & BD program (SS100001).

References