Rev Iberoam Micol. 2012;29(1):20–23

Revista Iberoamericana de Micología

www.elsevier.es/reviberoammicol

Original article

Antimicrobial activity of [2-(methacryloyloxy)ethyl]trimethylammonium chloride against *Candida* spp.

Cheila Denise Ottonelli Stopiglia[a], Fabrício Mezzomo Collares[b], Fabrício Aulo Ogliari[c], Evandro Piva[c], Carmen Beatriz Borges Fortes[b], Susana Maria Werner Samuel[b], Maria Lúcia Scroferneker[a,d,*]

[a] *Graduate Program in Medicine, Medical Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil*
[b] *Department of Conservative Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil*
[c] *Biomaterials Development and Control Center, School of Dentistry, Universidade Federal de Pelotas, Rio Grande do Sul, Brazil*
[d] *Department of Microbiology, Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil*

ARTICLE INFO

Article history:
Received 28 April 2010
Accepted 7 March 2011
Available online 5 April 2011

Keywords:
[2-(methacryloyloxy)ethyl]trimethylammonium chloride
Susceptibility
Candida spp.

ABSTRACT

Background: *Candida*-associated denture stomatitis is the most common manifestation of oral candidal infection, caused mainly by *Candida albicans*. Several authors have attempted to add antifungal agents or antiseptics to denture temporary soft lining materials or to denture acrylic resins, without relevant results. Therefore, the investigation of a quaternary ammonium functionalized compound [2-(methacryloyloxy)ethyl]trimethylammonium chloride (MADQUAT), which copolymerizes with methacrylates and which could act as a fungal inhibitor, is of paramount importance.
Aims: To evaluate the in vitro activity of MADQUAT against *Candida* species.
Methods: Thirty-one *Candida* strains were used to determine the in vitro antifungal activity of this compound. The minimum inhibitory concentrations and minimum fungicidal concentrations of MADQUAT and nystatin were determined.
Results: MADQUAT showed antifungal properties at concentrations of 6.25 to > 100 mg/ml, and fungicidal activity between 25 and > 100 mg/ml. The quantitative determinations of the fungistatic and fungicidal activity of MADQUAT showed fungistatic activity against all *Candida albicans*, *Candida krusei* and *Candida parapsilosis* strains, revealing fungicidal activity against some strains of the other species.
Conclusions: MADQUAT has antifungal activity against *Candida* spp. Moreover, the sensitivity to this substance varies across the different species in terms of MIC values and fungicidal or fungistatic activity.

Actividad antimicrobiana del cloruro de 2-metacriloil oxietil trimetilamonio contra *Candida* spp.

Palabras clave:
2-metacriloil oxietil trimetilamonio
Sensibilidad
Candida spp.

RESUMEN

Antecedentes: La estomatitis protética es la forma más común de infección bucal producida por especies de *Candida*, siendo *Candida albicans* el agente etiológico más común. Diversos autores han intentado asociar agentes antifúngicos o antisépticos a los materiales de revestimiento blando o a las resinas acrílicas de las prótesis dentales, pero sin éxito. Por ello, se ha investigado un compuesto de amonio cuaternario (2-metacriloil oxietil trimetilamonio [MADQUAT]), que copolimeriza con los metacrilatos y que podría actuar como inhibidor de levaduras.
Objetivos: El objetivo de este estudio fue evaluar la actividad in vitro del MADQUAT contra especies de *Candida*.
Métodos: Se utilizaron 31 cepas de *Candida* para determinar la actividad antifúngica in vitro. Se determinó la concentración mínima inhibitoria (CMI) y la concentración mínima fungicida del MADQUAT, así como de la nistatina.

* Corresponding author.
E-mail address: scrofern@ufrgs.br (M.L. Scroferneker).

1130-1406/$ – see front matter
doi:10.1016/j.riam.2011.03.003

Resultados: El MADQUAT presentó propiedades antifúngicas en las concentraciones entre 6,25 y > 100 mg/ml y actividad fungicida entre 25 y > 100 mg/ml. Los estudios cuantitativos de la actividad fungistática y fungicida del MADQUAT demostraron actividad fungistática contra todas las cepas de *Candida albicans*, *Candida krusei* y *Candida parapsilosis,* revelando actividad fungicida contra algunas cepas de otras especies.

Conclusiones: El MADQUAT presenta actividad antifúngica contra *Candida* spp. Además, la sensibilidad a dicho compuesto es distinta entre las diferentes especies considerando los valores de la CMI y la actividad fungicida o fungistática.

Candida is both a normal commensal and opportunistic pathogen found in warm-blooded animals, including humans. It colonizes the mucosal surfaces of the oral cavity, digestive or genitourinary tract of healthy individuals and causes a variety of infections depending on host susceptibility.[6] The prevalence of candidosis has increased due to the larger number of immunocompromised patients, including those on broad-spectrum antibacterial drugs, transplant recipients, and HIV-infected individuals[21] and, therefore, fungal infections have been given a lot of attention. One of the first clinical manifestations of candidosis occurs in the oral cavities of prosthesis (acrylic denture) wearers.

Candida species are found in the oral cavity of 25-50% of healthy individuals, in both adults and children. In denture wearers, these rates climb to 60-100%. *Candida albicans* is the most common species, accounting for almost 70% of the isolates. In addition to *Candida albicans*, other species including *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, *Candida guilliermondii*, and *Candida parapsilosis* are also usually isolated from denture and non-denture wearers.[27]

Candida adheres directly or via an intermediate layer of plaque-forming bacteria to denture acrylic resin (polymethylmethacrylate).[8] Despite antifungal treatment for denture stomatitis, infection recurs soon afterwards, suggesting that denture plaque may serve as a protected reservoir for *C. albicans*.[7] Several antifungal substances, such as triclosan, nystatin[3] and zeolite,[23] have been added to denture acrylic resins in order to avoid *Candida* proliferation on prosthetic device surfaces.

However, the addition of these substances to acrylic resins leads to short-term efficacy due to the leachability of the antifungal agent from the bulk of the polymer. Therefore, the investigation of a quaternary ammonium functionalized compound [2-(methacryloyloxy)ethyl]trimethylammonium chloride –MADQUAT– (fig. 1), which copolymerizes with methacrylates and which could act as a fungal inhibitor, is of paramount importance.

The aim of this study was to evaluate the activity of MADQUAT against *Candida* species, determining the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC).

Material and methods

Thirty-one *Candida* strains were used to determine the in vitro antifungal activity: *C. albicans* (*American Type Culture Collection* - ATCC 10231, ATCC 18804, ATCC 28367, 0050-L, 0051-L, MG), *C. dubliniensis* (22, 23, 25, 27, 28, 29, ATCC 7987), *C. glabrata* (0030-L, 993, ATCC 2001, MG), *C. krusei* (ATCC 6258, ATCC 20298, 0037-L, 219, 990, MG), *C. parapsilosis* (ATCC 22019, 0052-L, 0053-L, 0054-L, MG) and *C. tropicalis* (0056-L, ATCC 750, 0055-L).

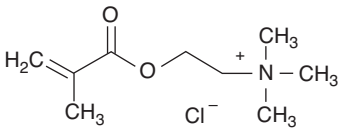

Figure 1. Chemical structure of [2-(methacryloyloxy)ethyl]trimethylammonium chloride.

Minimum inhibitory concentrations of MADQUAT and nystatin were determined using the *Clinical and Laboratory Standards Institute* M27-A3 methodology.[4] The strains were subcultured onto Sabouraud dextrose agar at 35 °C for 24 h. The inoculum was suspended in saline solution and adjusted to a final concentration of 0.5 x 10^3–2.5 x 10^3 in RPMI 1640 medium (Sigma, St Louis, MO, USA) buffered to pH 7.0 with 165 mmol l^{-1} morpholinopropanosulfonic acid (MOPS; Sigma).

Nystatin (Jansen-Cilag) was used as positive control. Stock solutions of nystatin and MADQUAT were prepared in dimethyl sulfoxide (DMSO; Vetec) and diluted in RPMI 1640 medium. The final concentrations of the antifungal agents ranged from 0.0312 to 16 μg/mL for nystatin and from 0.20 to 100 mg/ml for MADQUAT.

Sterilized round-bottomed 96-well microtiter plates (Cral Plast) were used, with addition of 100 μl of each drug to columns 1 to 10; 100 μl of RPMI 1640 medium were added to columns 11 and 12, which were used as growth positive and medium sterile controls, respectively. Aliquots of 100 μl of the standardized inoculum were added to the wells and the microtiter plates were incubated at 35 °C for 24 h. After incubation, the MIC was determined visually by comparison with the drug-free growth control well. The MIC was defined as the lowest concentration of the antifungal agent preventing visible fungal growth.

In order to determine the MFC, 100 μl of all wells with 100% of growth inhibition were seeded into culture tubes with 2 ml of Sabouraud dextrose broth medium. The tubes were incubated at 35 °C for 3 days to determine fungal growth. The MFC was the minimum fungistatic concentration that prevented fungal growth.[10]

Results and discussion

According to table 1, MADQUAT showed antifungal activity with a geometric mean (GM) MIC of 48.9 mg/ml (230 μM) whereas nystatin had a GM antifungal activity of 1.8 μg/ml. The quantitative determinations of the fungistatic and fungicidal activity of MADQUAT showed fungistatic activity against all *C. albicans*, *C. krusei* and *C. parapsilosis* strains, revealing fungicidal activity against some microorganisms of the other species. The nine reference strains of *Candida* spp. used in the present study demonstrated fungistatic activity (table 2). Nystatin showed fungicidal activity against all strains (GM 4.0 μg/ml).

Candida species frequently cause oral infections, including denture-related stomatitis, a chronic inflammatory condition associated with the oral mucosa and that affects 30-60% of patients wearing removable dental prostheses.[28] While several factors have been implicated in the etiology of denture stomatitis, such as poor denture hygiene, mechanical irritation, diet, use of antibiotics or allergic reaction to denture base material, *Candida* species have been recognized as the primary agents.[18,34]

Table 1
Range of antifungal activities of [2-(methacryloyloxy)ethyl]trimethylammonium chloride and nystatin against clinical isolates of *Candida* spp.

Strain	MADQUAT (mg/ml)		Nystatin (μg/ml)	
	MIC	MFC	MIC	MFC
C. albicans (6)				
Range	25-100	> 100	1-2	2-4
GM	50.0	> 100	1.8	3.6
C. dubliniensis (7)				
Range	12.5-100	50-> 100	1-2	2-8
GM	62.9	90.6	1.6	3.3
C. glabrata (4)				
Range	6.25-100	25-> 100	1-2	2-8
GM	35.4	59.5	1.4	4.8
C. krusei (6)				
Range	50-> 100	> 100	2-4	4-8
GM	63.0	> 100	2.4	4.5
C. parapsilosis (5)				
Range	50-100	> 100	2	4-8
GM	57.4	> 100	2	5.3
C. tropicalis (3)				
Range	25-50	50-> 100	2	2-4
GM	39.7	63.0	2	3.2
Over all (31)				
Range	6.25-> 100	25-> 100	1-4	2-8
GM	48.9	87.4	1.8	4.0

GM: geometric mean; MFC: minimum fungicidal concentration; MIC: minimum inhibitory concentration; (): number of isolates.

Microbial growth ensues from the adhesion of microbial cells to rough surfaces and from adhesive interactions between *Candida* species and oral bacteria, mostly streptococci.[28,29] The adhesion of *Candida* cells is a critical step in the colonization of human mucosal surfaces, on which the yeast lives as a commensal, causing disease whenever an opportunity arises. In addition, fungal adhesins are recognized virulence factors that contribute to pathogenesis.[2]

Several authors have attempted to add antifungal agents (nystatin, miconazole) or antiseptics (zeolite, triclosan) to denture temporary soft lining materials or to denture acrylic resins, but almost to no avail.[3,7,9,23] Quaternary ammonium compounds are age-old and well-known antiseptics with a favorable safety profile, and have been added to a variety of personal hygiene products. These compounds have shown MIC values between 0.25 and > 12,800 μg/ml against some bacteria such as *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*.[13,30,32] Other compounds showed microbiological activity against bacteria, but did not inhibit *C. albicans*.[25,30] However, several bisquaternary ammonium salts were investigated against *C. albicans*, showing 50% growth inhibition (GI50) values between 2.2 and > 350 μM. The most potent compound

Table 2
Antifungal activities of [2-(methacryloyloxy)ethyl]trimethylammonium chloride and nystatin against reference strains of *Candida* spp.

Strain	MADQUAT (mg/ml)		Nystatin (μg/ml)	
	MIC	MFC	MIC	MFC
C. albicans ATCC 10231	50	> 100	2	4
C. albicans ATCC 18804	50	> 100	2	4
C. albicans ATCC 28367	25	> 100	2	4
C. dubliniensis ATCC 7987	100	> 100	2	4
C. glabrata ATCC 2001	100	> 100	2	4
C. krusei ATCC 6258	50	> 100	2	4
C. krusei ATCC 20298	50	> 100	2	4
C. parapsilosis ATCC 22019	50	> 100	2	8
C. tropicalis ATCC 750	50	> 100	2	2

MFC: minimum fungicidal concentration; MIC: minimum inhibitory concentration.

–1,12-bis(tributylammonium)dodecane dichloride– contained a tributyl head group which seemingly has a direct effect on biological activity, considering that unsubstituted compounds have lower activity. Conversely, the compound contained a trimethyl head group with a GI50 of 175 μM.[22] MADQUAT also contains this head group, but our results were more consistent as MADQUAT thoroughly inhibited microbial growth at 230 μM. Thus, it is possible to infer that the alkyl 2-(methacryloyloxy)ethyl chain of MADQUAT enhances antifungal activity compared to dodecane. Nevertheless, in order to improve the activity of this compound in the future, the trimethyl group of MADQUAT can be replaced with tributyl. Another important issue to be investigated is the ideal pH for use of this polymer. Tapia et al.[31] showed that clinical isolates treated with the same polymer had different susceptibility profiles at different pH levels. Thus, this aspect must also be evaluated.

Quaternary ammonium compounds also have intrinsic detergent and anti-adhesive properties, especially against gram-positive bacteria.[12,14,24,26] These products reduce surface tension and have stronger attraction for negatively charged surfaces such as bacteria. These characteristics promote the adsorption of these products onto bacterial surfaces. Their mode of action is not associated with surface activity only, and thus cytolytic damage is the primary lesion caused by such cationic surfactants and a major contribution towards cell death. Consequently, there is a well-established relationship between cytolytic action and surface tension.[19]

In the study carried out by Caillier et al.[1] on quaternary ammonium compounds with the lowest inhibition concentration, the MFC is equal to the MIC. In all other cases, MIC and MFC values are very similar, ranging from 14.3 to > 2,000 μM. According to these authors, the results are in perfect agreement with the mechanism of action of quaternary ammonium in the first phase of inhibition of cell multiplication with relatively weak concentration (MIC) followed by a second phase of eradication, with higher concentrations (MFC). Our results follow this trend for *C. tropicalis* and *C. glabrata*. Although *C. dubliniensis* showed MIC values greater than the GM obtained for all strains, MADQUAT exhibited fungicidal activity against this species. On the other hand, MADQUAT did not show any fungicidal activity against *C. albicans*, *C. krusei* and *C. parapsilosis* strains at the concentrations analyzed.

In the experiment carried out by Codling et al.,[5] a quaternary ammonium compound, myristamidopropyl dimethylamine –MAPD–, possibly induced plasma membrane damage in *C. albicans,* but did not induce lysis of spheroplasts. According to Vieira and Carmona-Ribeiro,[33] the mechanism of antifungal action of quaternary ammonium compounds, hexadecyltrimethylammonium bromide (CTAB) and dioctadecyldimethyl ammonium bromide (DODAB), does not involve fungal cell lysis, but a change in cell surface charge from negative to positive instead.

Probably, this different profile of antifungal activity exhibited by MADQUAT is related to its mechanism of action and to the structure-activity relationship. Two factors known to be important in determining the antimicrobial activity of insoluble polymeric ammonium salts are the positive charge (ammonium group) density and the length of the substituent chain.[15,16] High positive charge density is believed to enhance the interaction of the ammonium group with the cytoplasmic membrane, while the long substituent chain may increase the hydrophobicity of the quaternary group, strengthening the interaction with the cytoplasmic membranes.[15] Moreover, with an increased hydrophobic surface, less cell adhesion[17,20] and a lower long-term degradation[11] of polymers are expected. Therefore, more studies are necessary to elucidate the mechanism of action and implications for the polymer properties of MADQUAT.

Our data indicate that MADQUAT has antifungal activity against *Candida* spp. Moreover, the sensitivity to this substance varies

across the different species in terms of MIC values and fungicidal or fungistatic activity.

Financing

The authors thank *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES), *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) and *Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul* (FAPERGS) for the financial support.

References

1. Caillier L, de Givenchy ET, Levy R, Vandenberghe Y, Geribaldi S, Guittard F. Synthesis and antimicrobial properties of polymerizable quaternary ammoniums. Eur J Med Chem. 2009;44:3201–8.
2. Calderone RA, Fonzi WA. Virulence factors of *Candida albicans*. Trends Microbiol. 2001;9:327–935.
3. Chow CK, Matear DW, Lawrence HP. Efficacy of antifungal agents in tissue conditioners in treating candidiasis. Gerodontology. 1999;16:110–8.
4. Clinical Laboratory Standards Institute (CLSI). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; Approved Standard-Third Edition. CLSI document M27-A3; 2008.
5. Codling CE, Maillard JY, Russell AD. Aspects of the antimicrobial mechanisms of action of a polyquaternium and an amidoamine. J Antimicrob Chemother. 2003;51:1153–8.
6. Cowen LE, Anderson JB, Kohn LM. Evolution of drug resistance in *Candida albicans*. Annu Rev Microbiol. 2002;56:139–65.
7. Douglas WH, Walker DM. Nystatin in denture liners-an alternative treatment of denture stomatitis. Br Dent J. 1973;135:55–9.
8. Edgerton M, Scannapieco FA, Reddy MS, Levine MJ. Human submandibular-sublingual saliva promotes adhesion of *Candida albicans* to polymethylmethacrylate. Infect Immun. 1993;61:2644–52.
9. El-Charkawi H, el-Said EA, Safouh HM, el-Raghi N. Effect of addition antimicrobial agents to denture reliners. Egypt Dent J. 1994;40:785–90.
10. Favre B, Ghannoum MA, Ryder NS. Biochemical characterization of terbinafine-resistant *Trichophyton rubrum* isolates. Med Mycol. 2004;42:525–9.
11. Ferracane JL. Hygroscopic and hydrolytic effects in dental polymer networks. Dent Mater. 2006;22:211–22.
12. Gupta AK, Ahmad I, Summerbell RC. Fungicidal activities of commonly used disinfectants and antifungal pharmaceutical spray preparations against clinical strains of *Aspergillus* and *Candida* species. Med Mycol. 2002;40:201–8.
13. Hu Y, Du Y, Yang J, Kennedy JF, Wang X, Wang L. Synthesis, characterization and antibacterial activity of guanidinylated chitosan. Carbohyd Polym. 2007;67:66–72.
14. Imazato S, Ebi N, Takahashi Y, Kaneko T, Ebisu S, Russell RR. Antibacterial activity of bactericide-immobilized filler for resin-based restoratives. Biomaterials. 2003;24:3605–9.
15. Jiang S, Wang L, Yu HJ, Chen Y. Preparation of crosslinked polystyrenes with quaternary ammonium and their antibacterial behavior. React Funct Polym. 2005;62:209–13.
16. Kawabata N, Hayashi T, Matsumoto T. Removal of bacteria from water by adhesion to cross-linked poly(vinylpyridinium halide). Appl Environ Microbiol. 1983;46:203–10.
17. Klotz SA, Drutz DJ, Zajic JE. Factors governing adherence of *Candida* species to plastic surfaces. Infect Immun. 1985;50:97–101.
18. Manfredi M, Merigo E, Salati A, Conti S, Savi A, Polonelli L, et al. *In vitro* candidacidal activity of a synthetic killer decapeptide (KP) against *Candida albicans* cells adhered to resin acrylic discs. J Oral Pathol Med. 2007;36:468–71.
19. Merianos JJ. Disinfection, Sterilization and Preservation. In: Block SS, editor. Disinfection, Sterilization and Preservation. Philadelphia: Lea and Febiger; 1992.
20. Minagi S, Miyake Y, Inagaki K, Tsuru H, Suginaka H. Hydrophobic interaction in *Candida albicans* and *Candida tropicalis* adherence to various denture base resin materials. Infect Immun. 1985;47:11–4.
21. Molero G, Díez-Orejas R, Navarro-García F, Monteoliva L, Pla J, Gil C, et al. *Candida albicans*: genetics, dimorphism and pathogenicity. Int Microbiol. 1998;1:95–106.
22. Ng CKL, Obando D, Widmer F, Wright LC, Sorrell TC, Jolliffe KA. Correlation of antifungal activity with fungal phospholipase inhibition using a series of bisquaternary ammonium salts. J Med Chem. 2006;49:811–6.
23. Nikawa H, Yamamoto T, Hamada T, Rahardjo MB, Murata H, Nakanoda S. Antifungal effect of zeolite-incorporated tissue conditioner against *Candida albicans* growth and/or acid production. J Oral Rehabil. 1997;24:350–7.
24. Othman HF, Wu CD, Evans CA, Drummond JL, Matasa CG. Evaluation of antimicrobial properties of orthodontic composite resins combined with benzalkonium chloride. Am J Orthod Dentofacial Orthop. 2002;122:288–94.
25. Palluotto F, Carotti A, Casini G, Ferappi M, Rosato A, Vitali C, et al. Synthesis and antibacterial activity of 2-aryl-2,5-dihydro-3(3H)-oxo-pyridazino[4,3-b]indole-4-carboxylic acids. Il Farmaco. 1999;54:191–4.
26. Pilloni AP, Buttini G, Giordano B, Iovene MR, di Salvo R, Buommino E, et al. The *in vitro* effects of cetyltrimethylammonium naproxenate on oral and pharyngeal microorganisms of various ecological niches. J Periodontal Res. 1999;34:473–7.
27. Pires FR, Santos EB, Bonan PR, De Almeida OP, Lopes MA. Denture stomatitis and salivary *Candida* in Brazilian edentulous patients. J Oral Rehabil. 2002;29:1115–9.
28. Radford DR, Challacombe SJ, Walter JD. Denture plaque and adherence of *Candida albicans* to denture-base materials *in vivo* and *in vitro*. Crit Rev Oral Biol Med. 1999;10:99–116.
29. Radford DR, Sweet SP, Challacombe SJ, Walter JD. Adherence of *Candida albicans* to denture-base materials with different surface finishes. J Dent. 1998;26:577–83.
30. Struga M, Kossakowski J, Stefańska J, Zimniak A, Koziol AE. Synthesis and antibacterial activity of bis-[2-hydroxy-3-(1,7,8,9,10-pentamethyl-3,5-dioxo-4-aza-tricyclo[5.2.1.$0^{2,6}$]dec-8-en-4-yloxy)-propyl]-dimethyl-ammonium chloride. Eur J Med Chem. 2008;43:1309–14.
31. Tapia CP, Soto DM, Vergara LG, Alburquerque CO, Maccioni AR, Matamala AM, et al. Efecto antifúngico de quitosán de alto peso molecular en cepas de *Candida* sp aisladas de muestras clínicas. Rev Chil Infect. 2009;26:515–9.
32. Thorsteinsson T, Másson M, Kristinsson KG, Hjálmasrsdóttir MA, Hilmarsson H, Loftsson T. Soft antimicrobial agents: synthesis and activity of labile environmentally friendly long chain quaternary ammonium compounds. J Med Chem. 2003;46:4173–81.
33. Vieira DB, Carmona-Ribeiro AM. Cationic lipids and surfactants as antifungal agents: mode of action. J Antimicrob Chemother. 2006;58:760–7.
34. Wilson J. The aetiology, diagnosis and management of denture stomatitis. Br Dent J. 1998;185:380–4.