Antiproliferative Effects of a Series of Cyclic Imides on Primary Endothelial Cells and a Leukemia Cell Line

José A. Yunes[a], Angelo A. Cardoso[b], Rosendo A. Yunes[c], Rogério Corrêa[d], Fátima de Campos-Buzzi[d], and Valdir Cechinel Filho[d,*]

[a] Laboratório de Biologia Molecular, Centro Infantil Boldrini, Campinas, SP, Brazil
[b] Cancer Research Institute, Indiana University, School of Medicine, Indianapolis, IN, USA
[c] Curso de Pós-Graduação em Química, UFSC, Florianópolis, SC, Brazil
[d] Núcleo de Investigações Químico-Farmacêuticas (NIQFAR), Universidade do Vale do Itajaí (UNIVALI), Itajaí, SC, Brazil. E-mail: cechinel@univali.br

* Author for correspondence and reprint requests

Z. Naturforsch. **63c**, 675–680 (2008); received February 12/April 3, 2008

The present study describes the cytotoxic properties of a series of 15 cyclic imides observed against different endothelial cells and K562 leukemic cells. Initially, eight structurally unrelated compounds were evaluated against cultured bone marrow endothelial cells (BMEC) and human umbilical vein endothelial cells (HUVEC). Only two imides showed cytotoxic activity at 10 μM. In continuation of our screening, eight compounds, structurally related to the compound with the higher cytotoxic activity, were assayed against endothelial cells and the K562 leukemic cell line. All of these new compounds except two exhibited cytotoxic and antiproliferative activities at concentrations below 10 μM against BMEC and HUVEC, respectively. The K562 leukemia cell line was only affected by concentrations of 100 μM. Preliminary SAR analysis indicated that the cytotoxic activity of these compounds was related to the presence of a planar imide ring directly bound to an aromatic ring.

Key words: Cyclic Imides, Leukemia, Angiogenesis, Bone Marrow Stromal Cells

Introduction

Cyclic imides belong to an important class of compounds due to their variety of biological properties (Hargreaves *et al*., 1970; Cechinel-Filho *et al*., 2003). Our research group has focused the attention on these compounds since the discovery of phyllanthimide, an alkaloid present in low concentration in the active aerial parts of *Phyllanthus sellowianus* (Tempesta *et al*., 1988). Using this compound as a model, we have synthesized a great number of compounds belonging to the different sub-classes of cyclic imides, including maleimides, succinimides, glutarimides, naphthalimides, and analyzed their different kinds of biological properties, such as antispasmodic, antibacterial, antifungal, and analgesic or antinociceptive effects (Cechinel-Filho and Yunes, 1998; Cechinel-Filho *et al*., 2003; Campos-Buzzi *et al*., 2003).

Extending our research program related to the biological properties of cyclic imides, we have now evaluated some of them regarding their potential use as antiangiogenic therapeutic agents. Cyclic imides are analogues of thalidomide, a compound known for its teratogenic effects, which has lately been shown to inhibit angiogenesis in experimental models (Stephens *et al*., 2000). The antiangiogenic and immunomodulatory properties of thalidomide have led to its evaluation in several clinical studies for the treatment of cancer (Fine *et al*., 2000; Singhal *et al*., 2000; Hwu *et al*., 2003). The second generation of thalidomide analogues with more potent antiangiogenic activities has been designed (Dredge *et al*., 2002).

The mechanism of action of thalidomide and its imide analogues is not completely understood. The antitumoural effect of thalidomide and its analogues may be mediated by direct inhibition of cancer cell survival and proliferation or by modulating the tumour microenvironment, for example, by inhibiting angiogenesis or enhancing the antitumoural immune system (Melchert and List, 2007).

Leukemia is associated with increased angiogenesis in the bone marrow (Aguayo *et al*., 2000), which is suspected to promote leukemia cell survival (Scappaticci *et al*., 2001; Iversen *et al*., 2002; Noren-Nystrom *et al.,* 2003). In this study we sought to characterize the cytotoxic activity of several synthetic cyclic imides against endothelial cells and leukemia.

0939–5075/2008/0900–0675 $ 06.00

Material and Methods

General chemical procedures

Solvents and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Aldrich (Steinheim, Germany), and were purified in the usual manner. Melting points were determined with a Microquimica AP-300 apparatus (Florianópolis, SC, Brazil) and were uncorrected. IR spectra were recorded with a Perkin Elmer 720 spectrometer (Perkin-Elmer, Norwalk, CT, USA) on KBr disks. The ^{13}C and ^{1}H NMR spectra were recorded on a Bruker 200 MHz instrument (Bruker, Karlsruhe, Germany). Elemental analyses were determined using a Perkin Elmer 2400 instrument. Percentages of C and H were in agreement with the product formula (within ± 0.4% of theoretical values). Compounds were dissolved in deuterated solvents from commercial sources (Sigma Chemical Co.) with tetramethylsilane (TMS) as the internal standard. Compounds were purified by recrystallization with the appropriate solvent or by column chromatography on SiO_2 (Merck, Darmstadt, Germany)

Synthesis of imides

Cyclic imides studied were obtained as previously described by our research group with yields ranging from 40 to 98%. 4-Aminoantipyrine derivatives **1** and **2** were synthesized as previously described (Cechinel-Filho *et al*., 1998; Campos *et al*., 2002). *N*-Phenyl and *N*-arylalkylmaleimides **3**–**6**, **8**–**11** were obtained by the reaction of maleic anhydride with substituted anilines or appropriate alkyl anilines in diethyl ether and dehydration of the corresponding maleamic acid (similar to **12**) by treatment with hot acetic anhydride/sodium acetate or acetic acid under reflux, as previously described (Cechinel-Filho *et al*., 1994, 1996). *N*-Phenyldichloromaleimide (**7**) was obtained by the reaction of dichloromaleic anhydride and aniline under reflux for 2 h with acetic acid (Andricopulo *et al*., 1998). Glutarimide derivative **13** was obtained according to Stiz and co-workers (2000), whereas tetrahydrophthalimide (**14**) and the acid analogue **15** were synthesized by reaction of *cis*-1,2,3,6-tetrahydrophthalic anhydride with appropriate amines (Costa *et al*., 2007).

Cell isolation and culture

Bone marrow endothelial cells (BMEC) were isolated from donor bone marrow samples using CD105 microbeads (Miltenyi Biotech, Auburn, CA, USA) according to the manufacturer's specifications. The CD105 positive cells were cultured in EGM2-MV medium (Cambrex BioScience, Walkersville, MD, USA) at 37 °C in 5% CO_2. Cells with 2 to 5 passages were used in the experiments. Human umbilical vein endothelial cells (HUVEC) were obtained from Cambrex BioScience and cultured in EGM2-MV medium. K562 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS).

MTS assay

For the cytotoxic assay thalidomide and various imide analogues were dissolved in DMSO to obtain a stock solution of 100 mM. Further dilutions were made in culture medium (RPMI 1640 supplemented with 5% FBS) immediately before use. K562 cells were plated at 25,000 cells/well in RPMI 1640 medium supplemented with 10% FBS. Cultured BMEC or HUVEC were trypsinized and 3,000 cells/well were plated in 96-well plates with 100 μL EGM2-MV medium. After overnight incubation, 25 μL of serial imide dilutions were added to each well, in triplicate, at final concentrations of 1, 10, and 100 μM. DMSO used as a vehicle was included as control at the final content of 0.1%. At definite incubation times, cell number and viability were measured by adding 25 μL of MTS reagent (Promega). After 1 h (for K562 cells) or 3 h (for BMEC and HUVEC) of incubation at 37 °C and 5% CO_2, the absorbance was measured at 490 nm and the cell number was calculated by comparison with a standard curve.

Results and Discussion

The activities of several cyclic imides (Fig. 1) belonging to different sub-classes were assayed against proliferation and survival of primary cultured endothelial cells by using the MTS colorimetric assay (Promega). As shown in Fig. 2, only compounds **1** and **6** showed any effect against the cells tested. At 10 μM compound **6** showed a significant inhibition of cell proliferation, whereas at 100 μM there was a strong cytotoxic effect. Proliferative inhibition was more prominent against bone marrow endothelial cells (BMEC) in comparison to human umbilical vein endothelial cells (HUVEC). Although these are preliminary re-

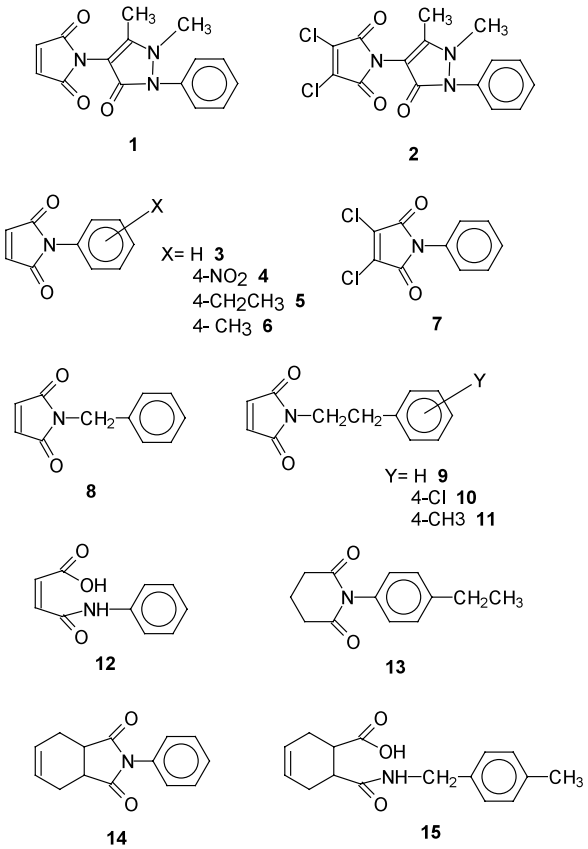

Fig. 1. Chemical structures of the cyclic imides studied.

sults, which must be interpreted with caution, such a "cell-specific" effect seems convenient to inhibit the marrow microenvironment while preserving the cardiovascular system. Compound **1** seemed to be less active than compound **6**, and again, it was more deleterious against BMEC than HUVEC.

Thalidomide, used here for the purpose of comparison, showed no activity in our *in vitro* assays (Fig. 2). It is well known that thalidomide has modest or negligible activity in most of the *in vitro* assays, compared to highly potent effects *in vivo*. This is consistent with the notion that thalidomide activity depends on some form of metabolic activation of the compound (Bauer *et al*., 1998). Some of the cyclic imides included in our study may as well require some form of metabolic activation.

It is important to mention that both more interesting compounds, **1** and **6**, previously exhibited biological effects which may support, at least partially, the findings described here. Compound **1** exerted antibacterial activity against *Staphylococcus aureus*, *S. epidermides* and *Escherichia coli* and both caused a drastic effect on the viability of peritoneal macrophages and B16-F10 melanoma cells, and caused strong inhibition of oxygen consumption (Prado *et al*., 2004). On the other hand, compound **6** also exhibited antifungal actions against *Candida albicans, Microsporum canis* and *Penicillium* sp. (Lima *et al*., 1999).

In continuation of our screening work, other compounds structurally related to compound **6** were assayed against endothelial cells and the K562 leukemic cell line. As shown in Fig. 3, all of them, except for compound **4** exhibited cytotoxic and antiproliferative activities at concentrations below 10 μM for BMEC and HUVEC, respectively. The K562 leukemia cell line was only affected by 100 μM. Compound **4** also showed some cytotoxic activity against BMEC and HUVEC, but concentrations required were around 100 μM and had no effect against K562 cells.

Important insights into the relation between biological activity and molecular structure are obtained from a preliminary SAR analysis. It is clear that the imidic and the aromatic rings must be directly bound, since any separation of methylene groups gives inactive compounds (compound **6** compared with compounds **8**–**11**). The imide ring should be planar that is with a double bond; the lack of this planarity gives inactive compounds (compare **6** with **13** and **14**). Finally, the substitution in the imide ring with chlorine atoms gives inactive compounds (compare **6** with **7**).

In conclusion, we were able to identify a series of cyclic imide compounds with putative antiangiogenic/antileukemic activities. Cytotoxic activity was related to a planar imidic ring directly bound to an aromatic ring.

Acknowledgements

This research was financially supported by CNPq grants to R. A. Y. and V. C. F.; J. A. Y. and A. A. C. were partially supported by NIH PO1 Grant CA 68484. J. A. Y. was partially supported by a grant from the Children's Leukemia Research Association (USA).

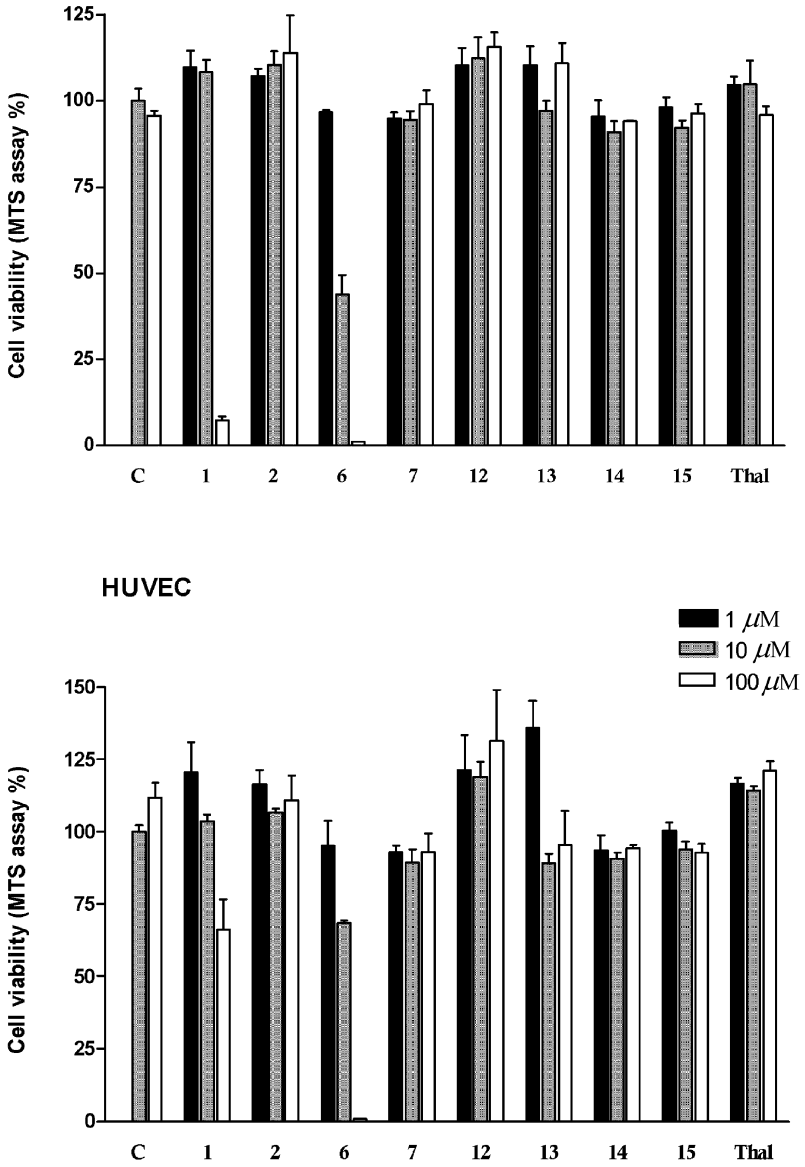

Fig. 2. Cytotoxic activity of eight different imides on the *in vitro* proliferation and survival of both bone marrow and umbilical vein endothelial cells. Imides were assayed against BMEC (primary bone marrow endothelial cells) and HUVEC (primary human umbilical vein endothelial cells). The number of viable cells was determined after 60 h of cultivation by the MTS colorimetric assay. The effect of thalidomide was included for comparison. Bars represent mean ± SD of cell viability relative to that obtained in culture medium alone. The experiment consisted of three measurements (three independent wells). Three different imide concentrations were assayed: 1, 10, and 100 μM. C, controls; 1, 2, 6, 7, 12–15, imides **1**, **2**, **6**, **7**, **12**–**15**; Thal, thalidomide. For imide structures see Fig. 1. The control grey bar corresponds to EGM2-MV culture medium. The control white bar corresponds to EGM2-MV culture medium with 0.1% DMSO.

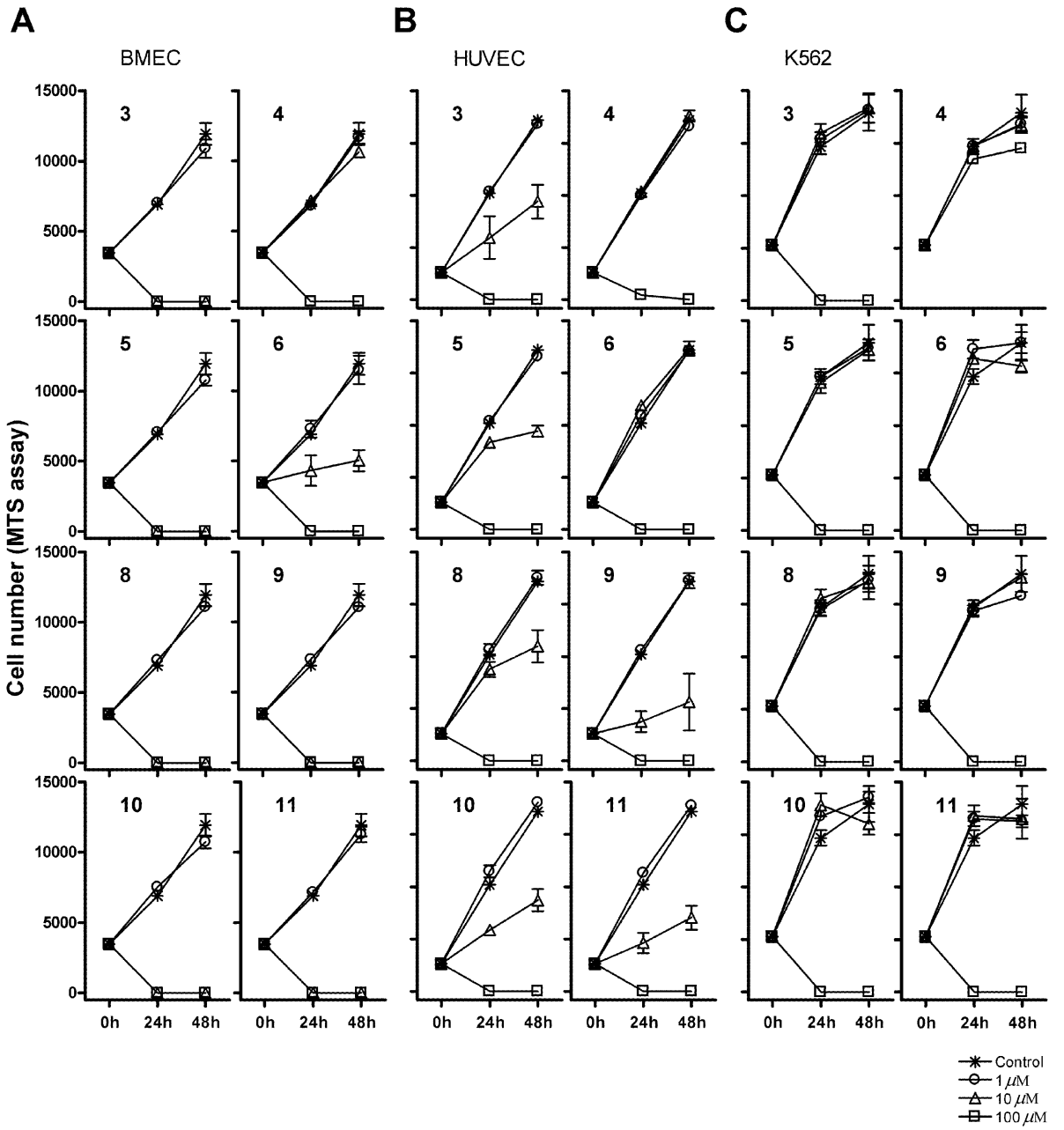

Fig. 3. Cytotoxic activity of eight different imides on the *in vitro* proliferation and survival of endothelial cells and the K562 leukemic cell line. Imides were assayed against three different cultured cells: (A) BMEC (primary bone marrow endothelial cells); (B) HUVEC (primary human umbilical vein endothelial cells); (C) K562, a cell line derived from chronic myeloid leukemia in blastic crisis. The number of viable cells was determined, after 24 h and 48 h of cultivation, by the MTS colorimetric assay. Each point represents the mean ± SD of the number of cells from three independent wells. *, control (EGM2-MV medium with 0.1% DMSO); ○, 1 μM; Δ,10 μM; □, 100 μM of imides **3**–**6**, **8**–**11**. For imide structures see Fig. 1.

Aguayo A., Kantarjian H., Manshouri T., Gidel C., Estey E., Thomas D., Koller C., Estrov Z., O'Brien S., Keating M., Freireich E., and Albitar M. (2000), Angiogenesis in acute and chronic leukemias and myelodysplastic syndromes. Blood **96**, 2240–2245.

Andricopulo A. D., Yunes R. A., Nunes R. J., Savi A. O. S., Corrêa R., Bella Cruz A., and Cechinel-Filho V. (1998), Síntese e atividade antibacteriana de imidas cíclicas: 3,4-dicloromaleimidas e 3-cloro-4-substituídamaleimidas. Quim. Nova **21**, 573–577.

Bauer K. S., Dixon S. C., and Figg W. D. (1998), Inhibition of angiogenesis by thalidomide requires metabolic activation, which is species-dependent. Biochem. Pharmacol. **55**, 1827–1834.

Campos F., Corrêa R., de Souza M. M., Yunes R. A., Nunes R. J., and Cechinel-Filho V. (2002), Studies on new cyclic imides obtained from 4-aminoantipyrine with analgesic properties. Potent effects of a 3,4-dichloromaleimide derivative. Drug Res. **52**, 455–461.

Campos-Buzzi F., Corrêa R., and Cechinel-Filho V. (2003), Síntese de moléculas bioativas: o exemplo das imidas cíclicas. In: Ciências Farmacêuticas: contribuição ao desenvolvimento de novos fármacos e medicamentos (Bresolin T. M. B. and Cechinel Filho V., eds.). Ed. UNIVALI, Itajaí-SC, Brazil, pp. 57–105.

Cechinel-Filho V. and Yunes R. A. (1998), Estratégias para obtenção de compostos farmacologicamente ativos a partir de plantas medicinais. Conceitos sobre modificação estrutural para otimização da atividade. Quim. Nova **21**, 99–105.

Cechinel-Filho V., Bella Cruz A., Moretto E., Pinheiro T., Nunes R. J., and Yunes R. A. (1994), Antibacterial activity of *N*-phenylmaleimides, *N*-phenylsuccinimides and related compounds. Structure-activity relationships. Farmaco **49**, 675–677.

Cechinel-Filho V., de Queiroz E. F., Lima E. O., Pinheiro T. R., Nunes R. J., and Yunes R. A. (1996), Síntese de *N*-alquilfenilmaleimidas e *N*-alquilarilmaleimidas com atividade antifúngica. Quim. Nova **19**, 590–593.

Cechinel-Filho V., Corrêa R., Vaz Z., Calixto J. B., Nunes R. J., Pinheiro T. R., Andricopulo A. D., and Yunes R. A. (1998), Further studies on analgesic activity of cyclic imides. Farmaco **53**, 55–57.

Cechinel-Filho V., de Campos F., Corrêa R., Yunes R. A., and Nunes R. J. (2003), Aspectos químicos e potencial terapêutico de imidas cíclicas: uma revisão da literatura. Quim. Nova **26**, 230–241.

Costa B. B. C., Corrêa R., de Souza M. M., Pretto J. B., Ardenghi J. V., Campos-Buzzi F., and Cechinel-Filho V. (2007), Antinociceptive effects of tetrahydro-phthalimides and related compounds. Z. Naturforsch. **62c**, 201–206.

Dredge K., Marriott J. B., Macdonald C. D., Man H. W., Chen R., Muller G. W., Stirling D., and Dalgleish A. G. (2002), Novel thalidomide analogues display anti-angiogenic activity independently of immunomodulatory effects. Br. J. Cancer **87**, 1166–1172.

Fine H. A., Figg W. D., Jaeckle K., Wen P. Y., Kyritsis A. P., Loeffler J. S., Levin V. A., Black P. M., Kaplan R., Pluda J. M., and Yung W. K. (2000), Phase II trial of the antiangiogenic agent thalidomide in patients with recurrent high-grade gliomas. J. Clin. Oncol. **18**, 708–715.

Hargreaves M. K., Pritchard J. G., and Dave H. R. (1970), Cyclic carboxylic monoimides. Chem. Rev. **70**, 439–469.

Hwu W. J., Krown S. E., Menell J. H., Panageas K. S., Merrell J., Lamb L. A., Williams L. J., Quinn C. J., Foster T., Chapman P. B., Livingston P. O., Wolchok J. D., and Houghton A. N. (2003), Phase II study of temozolomide plus thalidomide for the treatment of metastatic melanoma. J. Clin. Oncol. **21**, 3351–3356.

Iversen P. O., Sorensen D. R., and Benestad H. B. (2002), Inhibitors of angiogenesis selectively reduce the malignant cell load in rodent models of human myeloid leukemias. Leukemia **16**, 376–381.

Lima E. O., Queiroz E. F., Andricopulo A. D., Nunes R. J., Yunes R. A., Corrêa R., and Cechinel-Filho V. (1999), Evaluation of antifungal activity of *N*-arylmaleimides and *N*-phenylalkyl-3,4-dichloromaleimides. Bol. Soc. Chil. Quim. **44**, 185–189.

Melchert M. and List A. (2007), The thalidomide saga. Int. J. Biochem. Cell Biol. **39**, 1489–1499.

Noren-Nystrom U., Eriksson M., Eriksson B., Roos G., Bergh A., and Holmberg D. (2003), Antitumor activity of the angiogenesis inhibitor TNP-470 on murine lymphoma/leukemia cells *in vivo* and *in vitro*. Exp. Hematol. **31**, 143–149.

Prado S. R., Cechinel-Filho V., Campos-Buzzi F., Corrêa R., Cadena S. M., and de Oliveira M. B. (2004), Biological evaluation of some selected cyclic imides: mitochondrial effects and *in vitro* cytotoxicity. Z. Naturforsch. **59c**, 663–672.

Scappaticci F. A., Smith R., Pathak A., Schloss D., Lum B., Cao Y., Johnson F., Engleman E. G., and Nolan G. P. (2001), Combination angiostatin and endostatin gene transfer induces synergistic antiangiogenic activity *in vitro* and antitumour efficacy in leukemia and solid tumors in mice. Mol. Ther. **3**, 186–196.

Singhal S., Mehta J., Desikan R., Ayers D., Roberson P., Eddlemon P., Munshi N., Anaissie E., Wilson C., Dhodapkar M., Zeddis J., and Barlogie B. (2000), Antitumor activity of thalidomide in refractory multiple myeloma. N. Engl. J. Med. **341**, 1565–1571 (1999). Erratum in: N. Engl. J. Med. **342**, 364 (2000).

Stephens T. D., Bunde C. J., and Fillmore B. J. (2000), Mechanism of action in thalidomide teratogenesis. Biochem. Pharmacol. **59**, 1489–1499.

Stiz D., de Souza M. M., Golim V., Neto R. A. S., Corrêa R., Nunes R. J., Yunes R. A., and Cechinel-Filho V. (2000), Antinociceptive properties of *N*-aryl-glutaramic acids and *N*-aryl-glutarimides. Pharmazie **55**, 942–944.

Tempesta M. S., Corley D. G., Beutler J. A., Metral C. J., Yunes R. A., Giacomozzi C. A., and Calixto J. B. (1988), Phyllanthimide, a new alkaloid from *Phyllanthus sellowianus*. J. Nat. Prod. **3**, 617–618.