



ELSEVIER

SHORT COMMUNICATION

Antiproliferative effects of mitraphylline, a pentacyclic oxindole alkaloid of *Uncaria tomentosa* on human glioma and neuroblastoma cell lines

E. García Prado^a, M.D. García Gimenez^a, R. De la Puerta Vázquez^a,
J.L. Espartero Sánchez^b, M.T. Sáenz Rodríguez^{a,*}

^aDepartment of Pharmacology, Faculty of Pharmacy, University of Seville, cl Profesor García González n. 2, 41012 Seville, Spain

^bDepartment of Organic and Pharmaceutical Chemistry, Faculty of Pharmacy, University of Seville, Seville, Spain

Received 1 March 2006; accepted 7 December 2006

Abstract

Uncaria tomentosa inner bark extract is a popular plant remedy used in folk medicine to treat tumor and inflammatory processes. In this study, the anti-tumoral effects of its pentacyclic alkaloid mitraphylline were investigated. Furthermore, its growth-inhibitory and cytotoxic effects on glioma GAMG and neuroblastoma SKN-BE(2) cell lines were studied using cyclophosphamide and vincristine as controls. A colter counter was used to determine viable cell numbers, followed by application of the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt, colorimetric method to evaluate cell viability in this cytotoxicity assay. Micromolar concentrations of mitraphylline (from 5 to 40 μM) inhibited the growth of both cell lines. It inhibited the growth of the two cell lines studied in a dose-dependent manner. The IC_{50} values were 12.3 μM (30 h) for SKN-BE(2) and 20 μM (48 h) for GAMG, respectively. This action suggests that mitraphylline is a new and promising agent in the treatment of human neuroblastoma and glioma.

© 2007 Elsevier GmbH. All rights reserved.

Keywords: *Uncaria tomentosa*; Antiproliferative activity; Glioma; Neuroblastoma; Oxindole alkaloid

Introduction

Uncaria tomentosa (Willdenow ex Roemer and Schultes) DC. (Rubiaceae) is a Peruvian thorny liana, which is commonly known as “uña de gato” or “cat’s claw”. The aqueous and hydroalcoholic extracts from the bark are used in folk medicine for the treatment of many different health problems, including rheumatism, arthritis, gastrointestinal disorders, weakness, viral infections (including AIDS), skin impurities and as a contraceptive. It is well known that the primary use of

the plant is in the treatment of tumor and inflammatory processes (Heitzman et al., 2005).

Cat’s claw has been shown to contain many different chemical constituents, including quinovic acid glycosides, sterols, tannins, procyanidins, flavonoids, polyhydroxylated triterpenes and at least 17 different alkaloids (Aquino et al., 1990; De Matta et al., 1976; Ganzera et al., 2001; Kitajima et al., 2002, 2003; Montoro et al., 2004; Muhammad et al., 2001a, b).

There are two botanic chemotypes of this species. Most of the pharmacological activity has been attributed to the one which contains more pentacyclic (rather than tetracyclic) oxindole alkaloids (Philp, 2004). However, the studies have not tested the isolated pentacyclic oxindole alkaloid fractions.

*Corresponding author.

E-mail address: mtsaenz@us.es (M.T. Sáenz Rodríguez).

Numerous investigations on the immunomodulatory and antiinflammatory properties of different extracts have been reported. There is a very close relationship between the processes. The inhibition of the activation of nuclear factor- κ B (NF- κ B), which serves as an important regulator of host immune and anti-inflammatory responses (Aguilar et al., 2002), and tumor necrosis factor- α (TNF- α) inhibition tests have demonstrated the ability of *U. tomentosa* extracts to suppress chronic inflammation (Sandoval et al., 2000). The latter is considered the primary mechanism of the anti-inflammatory and immunomodulatory actions of this species. It has also been shown that cat's claw induces human endothelial cells to release a lymphocyte-proliferation-regulating factor (Wurm et al., 1998) and prolongs lymphocyte survival (Akesson et al., 2003).

On the other hand, no single study has been realized to prove the direct inhibition of brain tumor cell growth by cat's claw. The only study that exists to date is on the effect of different alkaloids from *U. tomentosa* on some leukemia cell lines (Bacher et al., 2006).

The aim of this study was therefore to demonstrate, using an MTS colorimetric method to evaluate cell viability, the antitumoral action of mitraphylline as compared to two well-known citostatic agents, cyclophosphamide and vincristine. We have used two human brain cell lines, neuroblastoma SKN-BE(2) and malignant glioma GAMG.

Materials and methods

Plant extract

The plant material was collected in the Peruvian forest and was provided by Dr. Carlos S. González. The plant extract was produced as follows: 500 g of *U. tomentosa* dried inner bark were treated with ammonium hydroxide and dichloromethane. After filtration, the obtained solution was concentrated in vacuo to yield a residue, which was dissolved in a chlorhydric acid solution (3%). Ammonium hydroxide and dichloromethane were added again. After concentration in vacuo, the purified alkaloid fraction was obtained as a brown residue and the yield was 0.1%.

Isolation and identification of a phytochemical

The dried residue of alkaloid fraction (0.5 g) was subjected to silica gel column chromatography followed by elution with different solvents. The isolated compound was obtained as a solid. EIMS and ^1H and ^{13}C NMR experiments were realized for its identification using an AVANCE 500 spectrophotometer. COSY-DQF, NOESY, (^1H - ^{13}C)-HSQC, (^1H - ^{13}C)-HMBC and

(^1H - ^{15}N)-HMBC were necessary too. The solvent used for NMR spectra was CDCl_3 .

Cell cultures

Two cell lines have been used, SKN-BE(2) neuroblastoma (Interlab Cell Line Collection [ICLC] CBA, Genova, Italy) and GAMG glioma (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ] Braunschweig, Germany). Cell lines were seeded in 75-cm² tissue culture flasks (Falcon, Heidelberg, Germany). Both were maintained in RPMI 1640 (neuroblastoma) and Dulbecco's MEM (glioma) supplemented with 10% heat-inactivated fetal bovine serum according to the culture conditions suggested by the DSMZ. The medium was renewed every two days and the cell cultures were incubated at 37 °C in a humidified atmosphere (95% air and 5% CO_2).

Drug treatments

Mitraphylline, a pentacyclic oxindole alkaloid from the inner bark of *U. tomentosa*, was dissolved in ethanol. In order to determine SKN-BE(2) and GAMG cell proliferation, different concentrations (5, 10, 20, 30 and 40 μM) were evaluated. Cyclophosphamide (Prasfarma, Barcelona, Spain) and vincristine (Ciclum Farma, Madrid, Spain) at the same doses were used as controls.

Proliferation assays

Cell proliferation was evaluated using the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), according to the manufacture's instructions (CellTiter 96 Aqueous One-Solution Cell Proliferation Assay, Promega Corporation, Madison, USA). Both cell lines were cultured for 4–5 days to let them grow in monolayers. Cells were harvested by trypsinization and cell viability. Trypan blue exclusion. Cells were quantified using a colter counter. The experiences were realized in 96-well plates, each well containing 10^4 cells in a total volume of 100 μl . The plates were inoculated with drugs and incubated, respectively, for the first doubling time, 30 h for SKN-BE(2) and 48 h for GAMG. After that, 20 μl of MTS reagent was added to each well. After 90 min, the absorbances of the samples were read at 492 nm in a multiscanner microplate reader (TECAN Spectra classic, Barcelona, Spain). The quantity of product, as measured by optical density, was indirectly proportional to the number of living cells. Each experimental condition was assayed in duplicate and all experiments were performed at least three times (Muñoz et al., 2005).

Statistical analysis

All measurements were evaluated statically using Student's *t*-test, taking $p \leq 0.05$ and 0.001 as significance.

Results

Isolation and identification of mitraphylline

From 500 mg of the dried residue of alkaloid fraction, 200 mg of an isolated compound was obtained as white crystals, by silica gel column chromatography, as 14–16 fractions (dichloromethane/methanol, 9.5:0.5). EIMS and ^1H NMR, ^{13}C NMR, COSY-DQF, NOESY, $(^1\text{H}-^{13}\text{C})$ -HSQC, $(^1\text{H}-^{13}\text{C})$ -HMBC and $(^1\text{H}-^{15}\text{N})$ -HMBC were all realized. All chemical and spectroscopic data were identical with literature reports (Laus et al., 1997; Muhammad et al., 2001a, b; Seki et al., 1993).

Effects of mitraphylline on in vitro proliferation of human glioma GAMG and neuroblastoma SK-N-BE(2) cell lines

Growth of the GAMG glioma and SKN-BE(2) human neuroblastoma was observed after the addition of the *U. tomentosa* isolated alkaloid mitraphylline, using different doses, at the first doubling time (Figs. 1 and 2). We observed that treatment of both cell lines with the alkaloid fraction resulted in a concentration-dependent cytotoxicity (Fig. 3).

For the human glioma GAMG, the mitraphylline concentration required for a 50% viability inhibition (IC_{50} value) was $20.05 \mu\text{M}$ for 48 h. This dose is smaller than that observed for the control cultures treated with cyclophosphamide and vincristine, 50.85 and $30.43 \mu\text{M}$, respectively.

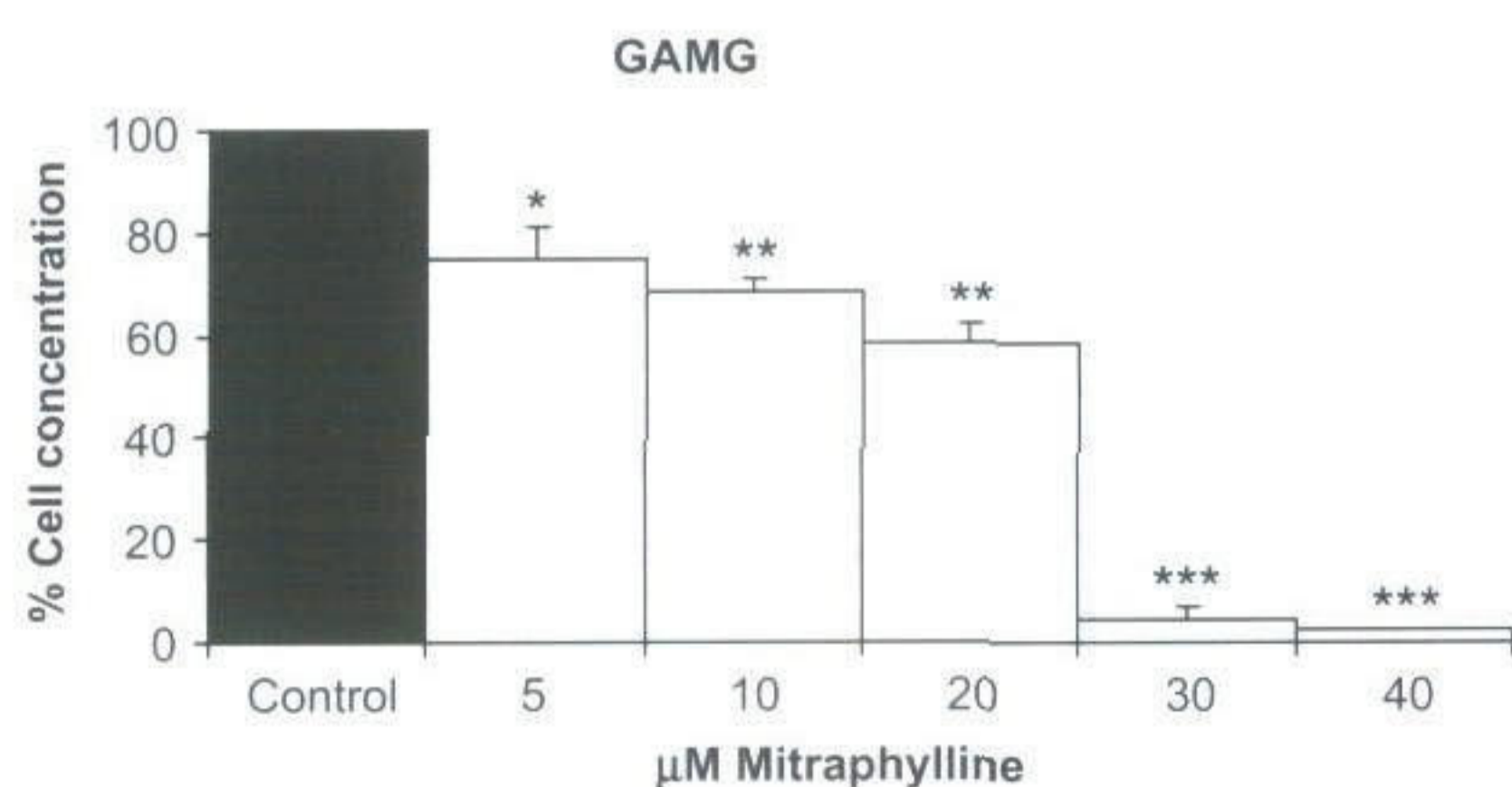


Fig. 1. Percentage of viability of GAMG cells at the first doubling time of incubation (48 h) in in vitro cultures after the treatment with increasing doses of mitraphylline. Values are the means \pm SD. The Student's *t*-test was measured vs. control. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

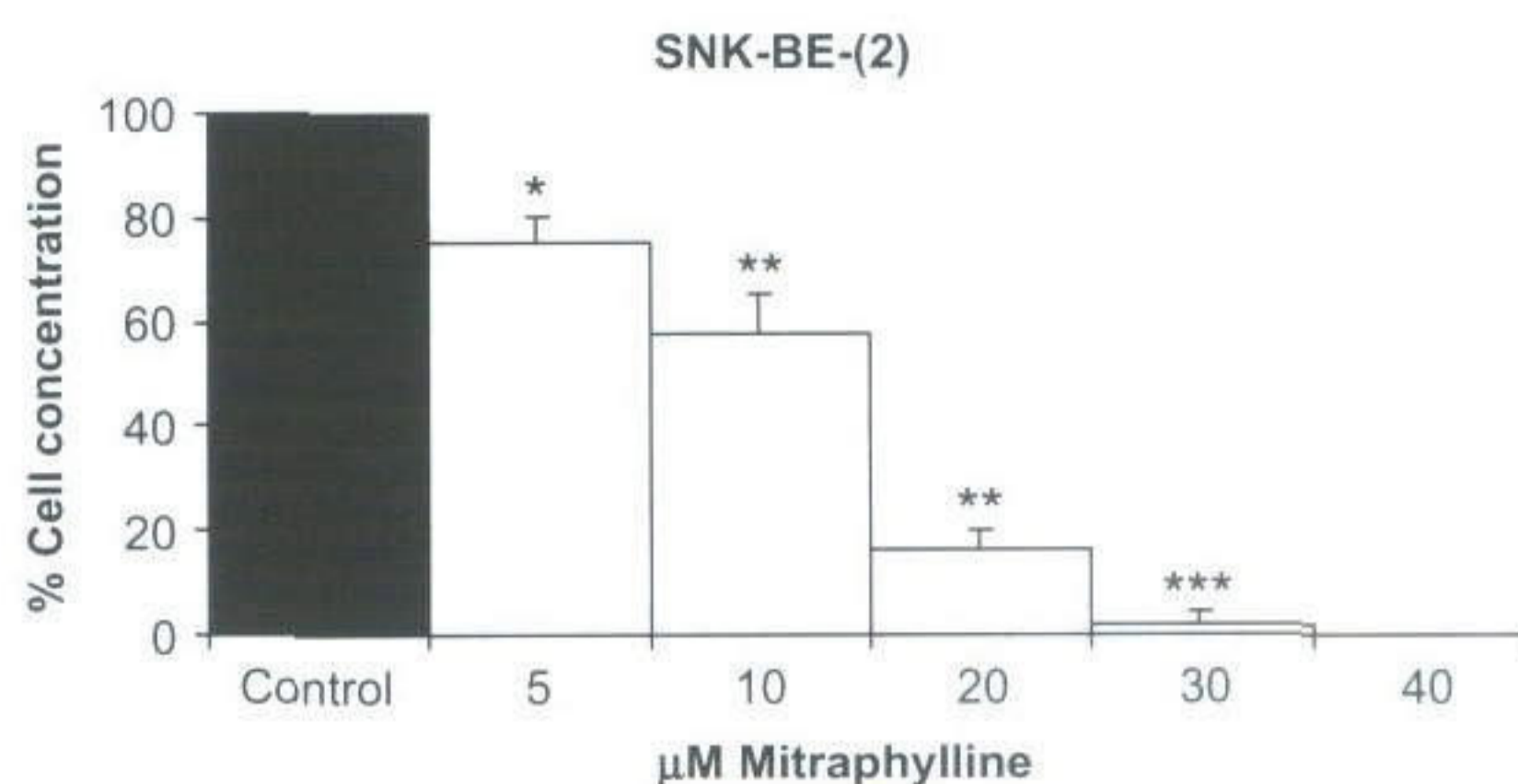


Fig. 2. Percentage of viability of SKN-BE(2) cells at the first doubling time of incubation (30 h) in in vitro cultures after the treatment with increasing doses of mitraphylline. Values are the means \pm S.D. The Student's *t*-test was measured vs. control. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

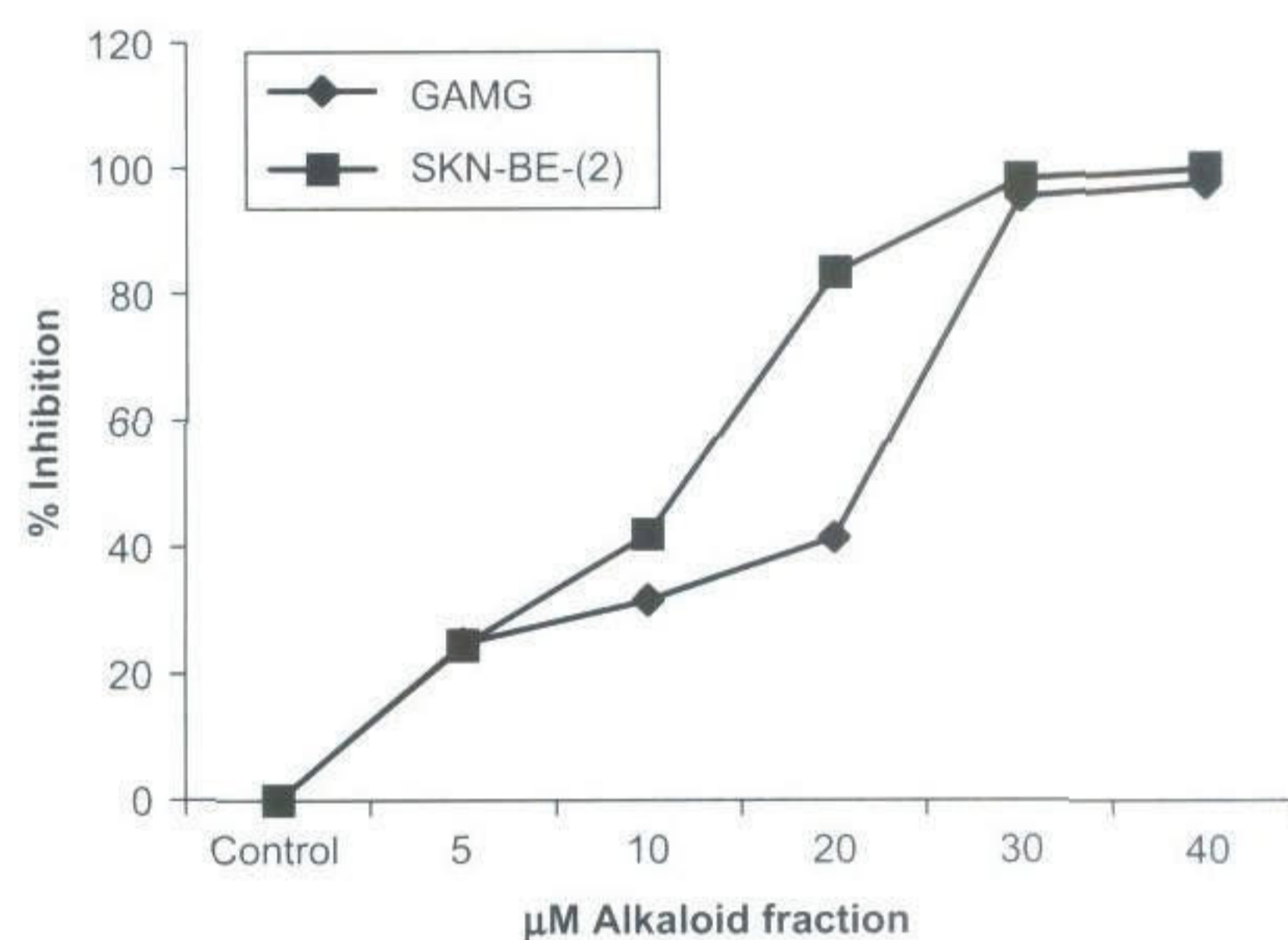


Fig. 3. Percentage of growth inhibition of GAMG and SKN-BE(2) cells at the first doubling time of incubation (48 and 30 h, respectively) in in vitro cultures after the addition of increasing concentrations of the alkaloid mitraphylline.

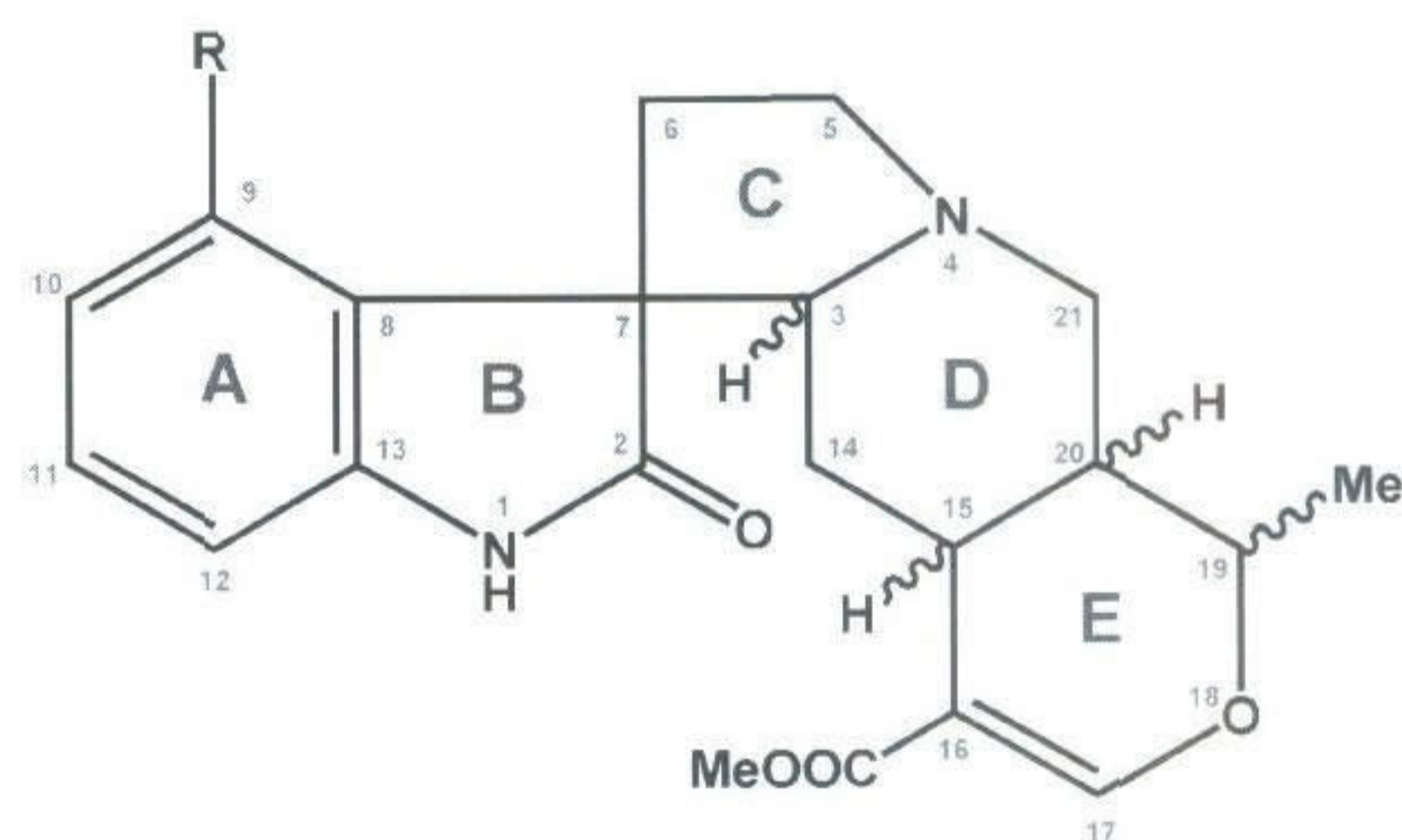


Fig. 4. General chemical structure of pentacyclic oxindole alkaloids.

For SKN-BE(2) human neuroblastoma, the IC_{50} value of the alkaloid was $12.28 \mu\text{M}$ for 30 h. The IC_{50} values for cyclophosphamide and vincristine were 8.05 and $10.05 \mu\text{M}$, respectively.

Table 1. IC₅₀ value data of mitraphylline and patrons, cyclophosphamide and vincristine, for GAMG glioma and SKN-BE(2) neuroblastoma at the first doubling time (48 and 30 h, in that order)

	Mitraphylline (μM)	Cyclophosphamide (μM)	Vincristine (μM)
GAMG	20.05	50.85	30.43
SKN-BE(2)	12.28	8.05	10.05

Maximum percentage inhibition was observed when the alkaloid fraction was assayed at 40 μM in both cases.

Discussion

A significant proportion of people in developing countries depend on folk medicine for the treatment of their health disorders. The use of *U. tomentosa* is well known in the treatment of tumor and inflammatory processes (Heitzman et al., 2005).

Previous to the proliferation assays, from the purified alkaloid fraction one main alkaloid of *U. tomentosa* bark was isolated and identified (Fig. 4). A complete chemical study of ¹H NMR, ¹³C NMR and 2D NMR spectra was required to obtain all the data necessary to identify the isolated compound, because of the difficulty if differentiating between the two isomers isomitraphylline and mitraphylline. All data were identical with literature reports for mitraphylline (Laus et al., 1997; Muhammad et al., 2001a, b; Seki et al., 1993; Wagner et al., 1985).

Following the identification of the alkaloid, its antitumoral effect was evaluated in vitro. In this paper, the potent antiproliferative effect of mitraphylline has been demonstrated in these two human brain tumor cell lines (glioma GAMG and neuroblastoma SKN-BE(2)). Our findings are also in agreement with the popular use of *U. tomentosa* and with reports of previous studies (Bacher et al., 2006).

Conclusions

Treatment with mitraphylline at μM concentrations produces growth inhibition and cell death in doses-dependent manner. In GAMG glioma line, the IC₅₀ value for mitraphylline is less than that obtained for cyclophosphamide and vincristine and very similar in SKN-BE(2) neuroblastoma, for the first doubling time (Table 1).

References

Aguilar, J.L., Rojas, P., Marcelo, A., Plaza, A., Bauer, R., Reininger, E., 2002. Anti-inflammatory activity of two

- different extracts of *Uncaria tomentosa* (Rubiaceae). *J. Ethnopharmacol.* 81, 271–276.
- Akesson, C., Pero, R.W., Ivars, F., 2003. C-Med 100, a hot water extract of *Uncaria tomentosa*, prolongs lymphocyte survival in vivo. *Phytomedicine* 10, 23–33.
- Aquino, R., De Simone, F., Vincieri, F.F., Pizza, C., 1990. New polyhydroxylated triterpenes from *Uncaria tomentosa*. *J. Nat. Prod.* 53, 559–564.
- Bacher, N., Tiefenthaler, M., Sturm, S., Stuppner, H., Ausserlechner, M., Kofler, R., 2006. Oxindole alkaloids from *Uncaria tomentosa* induce apoptosis in proliferating, G0/G1-arrested and bcl-2-expressing acute lymphoblastic leukaemia cells. *Br. J. Haematol.* 132, 615–622.
- De Matta, S.M., Monache, F.D., Ferrari, F., Marini-Bettolo, G.B., 1976. Alkaloids and procyanidins of a *Uncaria* sp. from Peru. *Pharmacology* 31, 527–535.
- Ganzera, M., Muhannad, I., Khan, R.A., Khan, I.A., 2001. Improved method for determination of oxindole alkaloids *Uncaria tomentosa* by high performance liquid chromatography. *Planta Med.* 67, 447–450.
- Heitzman, M.E., Neto, C.C., Winiarz, E., Vaisberg, A.J., Hammond, G.B., 2005. Ethnobotany, phytochemistry and pharmacology of *Uncaria* (Rubiaceae). *Phytochemistry* 66, 5–29.
- Kitajima, M., Hashimoto, K., Yokoya, M., Takayama, H., Sandoval, M., Aimi, N., 2003. Two new nor-triterpene glycosides from Peruvian “Uña de gato” (*Uncaria tomentosa*). *J. Nat. Prod.* 66, 320–323.
- Kitajima, M., Yokoya, M., Takayama, H., Aimi, N., 2002. Synthesis and absolute configuration of a new 3,4-dihydro-β-carboline-type alkaloid, 3,4-dehydro-5(S)-5-carboxystri-tosidine, isolated from Peruvian uña de gato (*Uncaria tomentosa*). *Chem. Pharm. Bull.* 50, 1376–1378.
- Laus, G., Brössner, D., Keplinger, K., 1997. Alkaloids of peruvian *Uncaria tomentosa*. *Phytochemistry* 45, 855–860.
- Montoro, P., Carbone, V., Quiroz, J., De Simone, F., Pizza, C., 2004. Identification and quantification of components in extracts of *Uncaria tomentosa* by HPLC-ES/MS. *Phytochemistry* 15, 55–64.
- Muhammad, I., Dunbar, D.C., Khan, R.A., Ganzera, M., Khan, I.A., 2001a. Investigation of Uña de Gato 1,7-Deoxyloganic acid and ¹⁵N NMR spectroscopic studies on pentacyclic oxindole alkaloids from *Uncaria tomentosa*. *Phytochemistry* 57, 781–785.
- Muhammad, I., Khan, I.A., Fischer, N.H., Fronczek, F.R., 2001b. Two stereoisomeric pentacyclic oxindole alkaloids from *Uncaria tomentosa*: uncarine C and uncarine E. *Acta Crystallogr. C* 57, 480–482.
- Muñoz, M., Rosso, M., Pérez, A., Coveñas, R., Rosso, R., Zamarrigo, C., Piruat, J.I., 2005. The NK1 receptor is involved in the antitumoral activity of L-733,060 and in the

- mitogenic action of substance P on neuroblastoma and glioma cell lines. *Neuropeptides* 39, 427–432.
- Philp, R.B., 2004. *Herbal–Drug Interaction and Adverse Effects. An Evidence-based Quick Reference Guide*. McGraw-Hill, New York, pp. 63–65.
- Sandoval, M., Charbonne, R.M., Okuhama, N.N., 2000. Cat's claw inhibits TNF-alpha production and scavenges free radicals: role in cytoprotection. *Free Rad. Biol. Med.* 29, 71–78.
- Seki, H., Takayama, H., Aimi, N., Sakai, S., Ponglux, D., 1993. A nuclear magnetic resonance study on the eleven stereoisomers of heteroyohimbine-type oxindole alkaloids. *Chem. Pharm. Bull.* 41 (12), 2077–2086.
- Wagner, H., Kreutzkamp, B., Jurcic, K., 1985. Die alkaloiden von *Uncaria tomentosa* und ihre phagozytose-steigernde Wirkung. *Planta Med.*, 419–423.
- Wurm, M., Kacani, L., Laus, G., Keplinger, K., Dierich, M.P., 1998. Pentacyclic oxindole alkaloids from *Uncaria tomentosa* induce human endothelial cells to release a lymphocyte-proliferation regulating factor. *Planta Med.* 64, 701–704.