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Influence of chelidonine, an inhibitor of tubulin polymerisation on tyrosine kinase activity in normal, transformed and malignant cell lines

Annie JOUBERT, Mona-Liza LOTTERING and Annie PANZER
Department of Physiology, University of Pretoria, P.O. Box 2034, Pretoria, 0001, South Africa
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ABSTRACT

Chelidonine, a tertiary hexahydro-benzophenanthridine alkaloid is an inhibitor of tubulin polymerisation and has been revealed to arrest cells in G_2/M. Since enhanced tyrosine kinase (TK) activity is linked to the transition from normal to the immortal malignant phenotype, the effect of 10 mM chelidonine was evaluated on TK activity in normal, transformed and malignant cell lines after 2 hours of exposure. Chelidonine caused a stimulation of TK activity in two normal cell lines (human foreskin fibroblast (Hs27) and normal monkey kidney (NMK)). In contrast, an inhibition of TK activity was observed in transformed human embryonic kidney (Graham 293) and transformed African green monkey kidney (Vero), as well as in human cervical carcinoma (HeLa) and squamous oesophageal carcinoma (WHCO5) cells. Hs27 cells exposed to chelidonine, revealed an increase in TK activity of 1.27-fold ($P < 0.05$). NMK cells showed a 1.15-fold increase in TK activity. A decrease in TK activity was observed in Graham 293 (0.91-fold) and Vero (0.45-fold) ($P < 0.005$) cells. In both HeLa and WHCO5 cells, the TK activity was reduced to 0.68-fold ($P < 0.05$) and 0.56-fold ($P < 0.005$) respectively. These data, including results from our previous studies, suggest a potential cross talk between the SAPK/JNK and TK signal transduction pathways and a possible differential effect of chelidonine on the phosphorylation status of role players involved in determining the length of G_2 arrest in normal versus transformed and malignant cells.

Chelidonine is a tertiary hexahydro-benzophenanthridine alkaloid, first isolated in 1839 from the plant *Chelidonium majus*. In 1942, this alkaloid was shown to cause mitotic arrest even before the discovery of tubulin (7). Biosynthesis of chelidonine from dopamine was reported in 1979 (1, 2, 18). It has since been revealed that chelidonine acts as a competitive inhibitor at the colchicine-binding site resulting in the inhibition of tubulin polymerisation (17, 23).

Interest in chelidonine was prompted when it was found to be a major contaminant of Ukrain™ (14), a compound reported to be selectively toxic to malignant cells (11, 21, 25). Chelidonine was proven was proven to be a weak inhibitor of cell growth when investigated in two normal (monkey kidney (NMK) and human foreskin fibroblast (Hs27)), two transformed (African green monkey kidney (Vero) and human embryonic kidney (Graham 293)) and two malignant (squamous oesophageal carcinoma (WHCO5) and human cervical carcinoma (HeLa)) cell lines (14). It was confirmed that chelidonine inhibits tubulin polymerisation (24 mM is required for 50% inhibition of tubulin polymerisation), resulting in a G_2/M arrest, characterised by abnormal metaphase morphology, increased levels of cyclin B1 and enhanced cell division cycle (cdc) 2 kinase activity (14, 23).

Since disruption of the microtubule network can be regarded as an intracellular stressor (6, 9), the stress-activated protein kinase/jun kinase (SAPK/

Correspondence to: Dr Annie Joubert, Dept of Physiology, PO Box 2034, 0001, PRETORIA, South Africa
Tel: +27 12 3192246, Fax: +27 12 3211679
e-mail: ajoubert@postillion.up.ac.za

JNK) pathway including Rac, cdc42, mitogen-activated protein kinase kinase kinase (MEKK), c-Jun NH_2-terminal kinase kinase (JNKK) and c-Jun NH_2-terminal kinase (JNK) is therefore strongly activated by spindle poisons (13). Chelidonine was also shown to activate the SAPK/JNK pathway *in vitro* (14).

In succession to our previous research where chelidonine's growth inhibitory effects and inhibition of tubulin polymerisation were confirmed *in vitro*, the aim of this study was to investigate the influence of chelidonine on protein tyrosine kinase (TK) activity in normal, transformed and tumour cell lines, since enhanced TK activity is linked to the transition from normal to the immortal malignant phenotype. Mitogenic signaling through the principal growth factor receptor tyrosine kinase (RTK) pathway, *i.e*. RTK, Ras, Raf, MAPK kinase and MAPK has been linked to the pathogenesis of human cancer (15). MAPK is important for transduction of extracellular stimuli to intracellular responses and can be activated by various extracellular signals, *e.g.* growth factors, cytokines and environmental stresses (9, 13). In addition, changes in phosphotyrosine proteins are vital for microtubule dynamics during sperm aster formation and mitotic spindle assembly (24).

MATERIALS AND METHODS

Cell lines. HeLa (human cervical carcinoma) and Hs27 (human foreskin fibroblast) cell cultures were purchased from the American Type Culture Collection (ATCC), Manassas, USA). Graham 293 (transformed human embryonic kidney) and Vero (transformed African green monkey kidney) cells were obtained from Highveld Biological (Sandringham, SA). Normal monkey kidney (NMK) cells, isolated from the kidneys of adult vervet monkeys, were kindly provided by the department of Virology (University of Pretoria, Pretoria, SA). The WHCO5 cell line was a gift from Professors Thornley and Veale (Department of Zoology, University of the Witwatersrand, Johannesburg, SA). WHCO5 cells were originally obtained through a biopsy specimen of a patient with squamous oesophageal carcinoma.

Cell culture maintenance and chemicals. The cells were grown and maintained as monolayer cultures in minimum essential medium, containing 10% heat-inactivated fetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2. No antibiotics were used. The final dimethyl sulphoxide (DMSO) concentration in the growth medium was 0.05% (v/v). Controls included showed that 0.05% DMSO had no effect on experiments conducted. Chelidonine (lot number 38H0621) was purchased from Sigma Chemical Co. (St. Louis, USA) and stored at 4°C until use. Chelidonine was freshly prepared in DMSO for each experiment. Eagle's minimum essential medium (EMEM) with Earle's salts, L-glutamine and $NaHCO_3$, Trypsin-EDTA, DMSO and Trypan blue were supplied by Sigma Chemical Co. (St. Louis, USA). Heat-inactivated fetal calf serum, sterile cell culture flasks and plates were obtained through Sterilab Services (Johannesburg, SA). Phosphate buffered saline (PBS) was purchased from Gibco BRL through Laboratory Specialist Services (Johannesburg, SA). Phenyl methyl sulfonyl fluoride (PMSF), leupeptin, aprotinin and RNase were supplied by Boehringer Mannheim (Mannheim, Germany). All other chemicals were of analytical grade and supplied by Sigma Chemical Co. (St. Louis, USA).

Chelidonine exposure of cells and measurement of tyrosine kinase activity. Total TK activity was measured by means of a photometric enzyme immunoassay using streptavidin-coated microtiter plates, a biotin-labeled substrate peptide and a phosphotyrosine antibody as described by the instruction manual of Boehringer Mannheim (Mannheim, Germany). The assay detects the protein TK activity by monitoring the transfer of the γ-phosphate group from ATP to a tyrosine residue of the biotin-labeled substrate peptide. Specificity is ensured since the anti-phosphotyrosine antibody does not cross-react with phosphoserine or phosphothreonine.

Cells from stock flasks were trypsinised, seeded at 5×10^5 per 25 cm^2 flask after Trypan blue exclusion and left for 24 hours. Subsequently, cells were exposed to 0.05% DMSO (control), or 10 mM chelidonine for 2 hours. We used 10 mM Chelidonine, since no dose-response curve could be found in growth inhibition studies (14) as well as in the inhibition of tubulin polymerisation (23) in dosages exceeding 10 mM. The experiment was terminated by lysing cells for 30 minutes at 0°C by gentle shaking with RIPA-buffer (300 ml, consisting of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 100 mg/ml PMSF, 1 mg/ml aprotinin, 2 mg/ml leupeptin and 100 mM Na_3VO_4). Lysates were transferred to micro tubes and centrifuged for 10 minutes at 10,000 × *g* at 4°C. Biotin-labeled substrate peptides were added to each sample. Protein tyrosine kinases present in the cell extract,

subsequently phosphorylated tyrosine residues of the biotin-labeled substrate peptides. The sample was then divided into triplicate wells of streptavidin-coated multi-well titer plates. Peroxidased anti-phosphotyrosine antibodies were added to each well. The anti-phosphotyrosine antibody does not cross-react with phosphoserine or phosphothreonine. After washing away unbound antibodies, ABTS® substrate for peroxidase was added. After 10 minutes of colour development, the absorbance of samples was measured at 405 nm, with reference wavelength at 450 nm on an EL_X800 Universal Microplate Reader (Biotek Instruments, Wilrijk, Belgium). Stimulation and inhibition of TK activity was determined in triplicate at reaction time 0, 15 and 30 minutes at 37°C. Independent replicates of experiments yielded similar results.

Calculation of tyrosine kinase activity. Protein TK activity was calculated as according to the method described (4, 19).

Statistics. Data obtained from independent experiments are shown as the mean ± SD and were statistically analysed for significance using the analysis of variance (ANOVA)-single factor model followed by a two-tailed Student's t-test. Means are presented in bar charts, with T-bars referring to standard deviations. Statistical significance of differences was evaluated with ANOVA. $P < 0.05$ was regarded as statistically significant.

RESULTS

The effect of 10 mM chelidonine was evaluated on TK activity relative to the DMSO-treated control cells in 6 cell lines after 2 hours of exposure to this alkaloid. TK activity was determined at reaction time 0, 15 and 30 minutes. TK activity in DMSO-treated cells is indicated by a solid line (Figs. 1–6). Chelidonine caused a stimulation of TK activity in the two normal cell lines (Hs27 and NMK) (Figs. 1 and 2) and an inhibition in the transformed (Graham 293 and Vero) (Figs. 3 and 4) and tumour (HeLa and WHCO5) (Figs. 5 and 6) cell lines. Fold increase or decrease in TK activity with respect to the DMSO-treated control cells was determined over 30 minutes in the linear region as described previously (4, 19). In Hs27 cells exposed to chelidonine, a statistically significant increase in TK activity of 1.27-fold was observed compared to the DMSO control cells (Fig. 1) ($P < 0.05$). NMK cells were less susceptible to TK stimulation compared to Hs27 and showed a 1.15-fold increase in TK activity when compared to DMSO-treated NMK cells (Fig. 2). Graham 293 cells exhibited a decrease of 0.91-fold in TK activity when compared to the DMSO-treated cells (Fig. 3). Vero cells were more susceptible to chelidonine than Graham 293 cells and revealed a statistically significant decrease of 0.45-fold in TK activity when compared to its control (Fig. 4) ($P < 0.005$). In both HeLa and WHCO5 cells, the TK activity was statistically significantly decreased 0.68-fold ($P < 0.05$) and 0.56-fold ($P < 0.005$) respectively compared to their DMSO controls (Figs. 5 and 6).

DISCUSSION

Disruption of microtubule dynamics can be regarded as an intracellular stressor resulting in the activation of the SAPK/JNK signal transduction pathway. Previous research has shown that chelidonine led to the activation of the SAPK/JNK signal transduction pathway in Hs27, NMK, Graham 293, Vero, HeLa and WHCO5 cell lines. Chelidonine reduced cell numbers, caused abnormal metaphases and inhibited tubulin polymerisation (14).

Since the mechanisms whereby chelidonine reduced cell numbers in the above-mentioned cells remain to be further explored, we investigated the effect of chelidonine on another signal transduction pathway, namely TK activity in these six cell lines. Chelidonine inhibited TK activity in transformed and malignant cell lines and enhanced TK activity in normal cell lines. Since enhanced TK activity is linked to the transition from normal to the immortal malignant phenotype (15) and since chelidonine led to a decrease in cell growth in HS27 and NMK cells, the increases observed in TK activity in the two normal cell lines after exposure to chelidonine were unexpected. It is unknown why TK activity is differentially modulated in normal cells compared to transformed or malignant cells. Differential modulation in normal, transformed and malignant cells has also been observed by Joubert *et al.* (4) in which free fatty acids stimulated TK activity in transformed and malignant cells, while they had no effect in normal cells. According to Iavarone *et al.* (3) environmental stresses as well as receptor tyrosine kinases regulate the activity of JNK. It is known that JNK phosphorylates Jun members of the activating protein-1 family of transcription factors thereby controlling cell growth, differentiation and apoptosis. The tyrosine kinase, c-abl, associates with retinoblastoma (Rb) protein, phosphorylates RNA polymerase

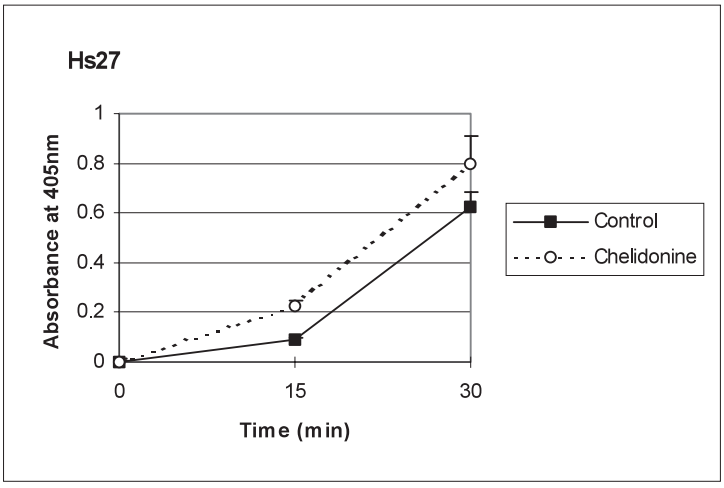

Fig. 1 The effect of 10 mM chelidonine on TK activity in Hs27 cells relative to the DMSO-treated control cells (indicated by a solid line) after 2 hours of exposure. TK activity was determined at reaction time 0, 15 and 30 minutes. In Hs27 cells exposed to chelidonine, a statistically significant increase in TK activity of 1.27-fold was observed compared to the DMSO control cells ($P < 0.05$).

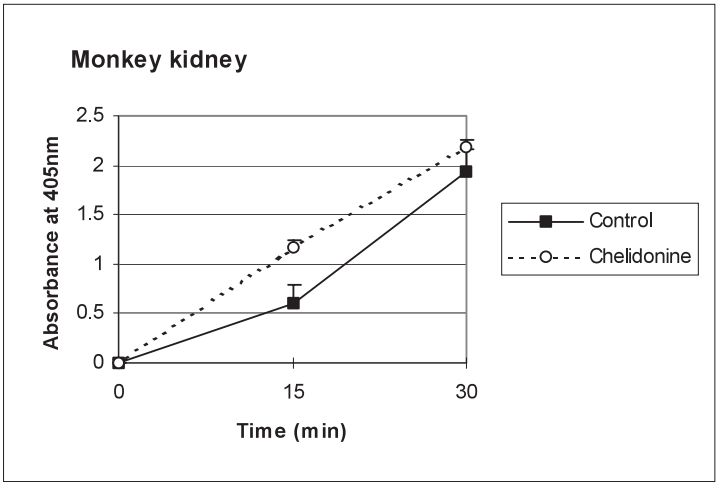

Fig. 2 The effect of 10 mM chelidonine on TK activity in NMK cells relative to the DMSO-treated control cells (indicated by a solid line) after 2 hours of exposure. NMK cells showed a 1.15-fold increase in TK activity when compared to DMSO-treated cells.

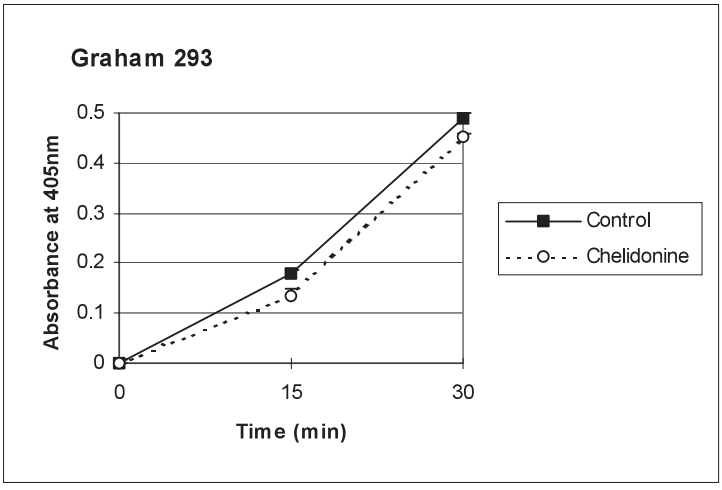

Fig. 3 The effect of 10 mM chelidonine on TK activity in Graham 293 cells relative to the DMSO-treated control cells (indicated by a solid line) after 2 hours of exposure. Graham 293 cells exhibited a decrease of 0.91-fold in TK activity when compared to the DMSO-treated cells.

II and stimulates transcription (12). However, overexpression of c-abl results in growth inhibition by causing a cell cycle arrest that is similar to that caused by p53 or Rb protein (12). The influence of chelidonine on the relation between receptor tyrosine kinases and the SAPK/JNK pathway remains to be elucidated.

We have previously shown that the tyrosine phos-

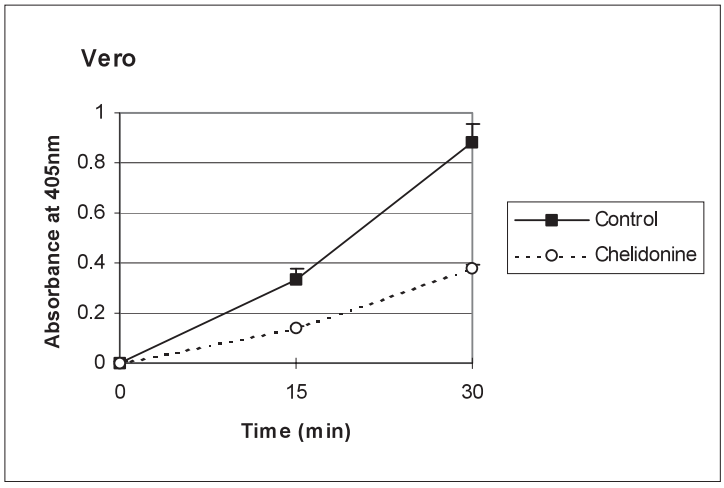

Fig. 4 The effect of 10 mM chelidonine on TK activity in Vero cells relative to the DMSO-treated control cells (indicated by a solid line) after 2 hours of exposure. Vero cells revealed a statistically significant decrease of 0.45-fold in TK activity when compared to its control ($P < 0.005$).

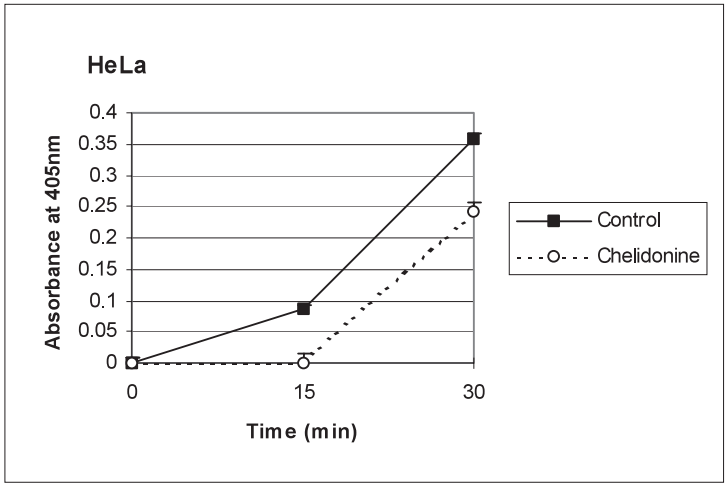

Fig. 5 The effect of 10 mM chelidonine on TK activity in HeLa cells relative to the DMSO-treated control cells (indicated by a solid line) after 2 hours of exposure. In HeLa cells the TK activity was statistically significantly decreased 0.68-fold ($P < 0.05$) compared to the DMSO controls.

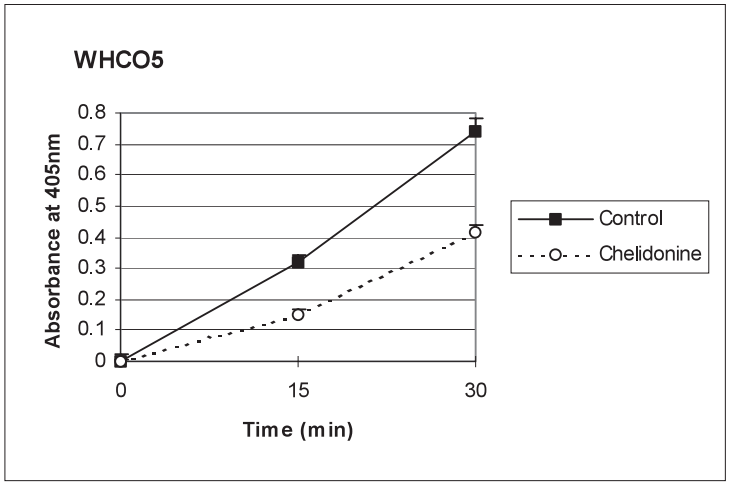

Fig. 6 The effect of 10 mM chelidonine on TK activity in WHCO5 cells relative to the DMSO-treated control cells (indicated by a solid line) after 2 hours of exposure. TK activity was statistically significantly decreased 0.56-fold ($P < 0.005$) compared to the DMSO controls.

phorylation status of a TK protein of approximately 55 kDa (~55 kDa) was influenced after exposure to prostaglandin A_2 (PGA_2) in two esophageal cancer cell lines (WHCO3 and WHCO1), human epithelial cervix carcinoma, MCF-7 and NMK cells. High levels of TK activity observed in cells exposed to PGA_2 were ascribed to the possible activation of a tyrosine dependent phosphatase that dephosphorylates the

~55 kDa protein resulting in a decrease of cell numbers (5).

Chelidonine has been revealed to arrest Hs27, NMK, Graham 293, Vero, HeLa and WHCO5 cell lines in G_2/M (14). Chelidonine caused an increase in cyclin B1 levels as well as an increase in cdc2 kinase activity leading to persistence of the spindle checkpoint and thus a prolonged metaphase arrest (14). Biochemical markers indicative of the G_2/M transition, include the activation of cyclin B1/cdc2 and the suppression of phosphorylation of cdc2 kinase on threonine 14 (Thr14) and tyrosine 15 (Tyr15) (22). Overexpression of the Wee1 protein and the phosphorylation of cdc2 kinase regulated by Wee1, may act to prolong the length of G_2 arrest (8). Thus, inhibition of cyclin-dependent kinases by Thr14/Tyr15 phosphorylation (located within the active site of the kinase) is critical for normal cell cycle progression and is a converging event for several cell cycle checkpoints (10, 16).

It has been shown that another microtubule damaging agent, podophyllotoxin, induced a G_2/M arrest in HT 29 cells by inhibiting normal mitotic spindle formation, increasing cyclin B1/cdc2 activity, increasing in cdc25 A phosphatase and cyclin dependent kinase 7 activity concomitantly, and well as down-regulating Wee1 protein expression (20).

As already mentioned, chelidonine also inhibited microtubule polymerisation, caused a G_2/M arrest, affected both the SAPK/JNK pathway (14) and TK activity (current study) in Hs27, NMK, Graham 293, Vero, HeLa and WHCO5 cell lines investigated. Additional studies are necessary to determine the downstream target molecules involved in, and the possible cross talk between the SAPK/JNK and TK signal transduction pathways. Since chelidonine inhibited TK activity in transformed and malignant cell lines and enhanced TK activity in normal cell lines, investigating the possible differential effects of chelidonine on Wee1 protein expression and the phosphorylation of cdc2 kinase regulated by Wee1 in these cell lines, will provide more insight into the action mechanism of this alkaloid on role players involved in determining the length of G_2 arrest.

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