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FEBS Letters 341 (1994) 99–103

FEBS 13757

FEBS LETTERS

Inhibition of Moloney murine leukemia virus replication by tyrphostins, tyrosine kinase inhibitors

Aflalo Esther, Seri Iftach, Priel Esther*

Department of Microbiology and Immunology, Faculty of Health Sciences, Ben-Gurion University of the Negev, P.O. Box 653, Beer-Sheva 8405, Israel

Received 29 December 1993; revised version received 27 January 1994

Abstract

We have previously shown that topoisomerase I (topo I) antagonist inhibited retrovirus replication. Since tyrphostins, synthetic compounds and protein tyrosine kinases (PTKs) blockers, inhibited topo I activity (manuscript in preparation) we examined their ability to inhibit Moloney murine leukemia virus (Mo-MuLV) replication. We found that non-cytotoxic doses of tyrphostin derivatives (AG-555, AG-18) blocked or substantially reduced Mo-MuLV replication in acute or chronically infected NIH/3T3 cells. Our experiments suggest that the antiviral effect of these tyrphostin derivatives was not the result of antiproliferative activity. However, the tyrphostin derivatives used in our present investigation differ in their ability to inhibit Mo-MuLV replication. Furthermore, as expected from stereospecific competitive inhibitors, the antiviral effect is not a general characteristic of all tyrphostin derivatives, since AG-213 does not affect Mo-MuLV replication. Our results indicate that these tyrphostin derivatives may represent a novel class of antiretroviral drugs.

Key words: Tyrphostin; Mo-MuLV replication; Antiretroviral drug

1. Introduction

Retroviruses have been etiologically implicated with leukemia in humans and animals. Human T-cell leukemia virus type 1 (HTLV-I) causes adult T-cell leukemia (ATL), particularly in high endemic areas of the virus such as Japan and the Caribbean [1] and human T-cell leukemia virus type 2 (HTLV-II) is involved in hairy cell leukemia [2]. Murine leukemia viruses, such as Moloney murine leukemia virus (Mo-MuLV), are classical replication competent retroviruses that lack oncogenes. They induce leukemias or other hematopoeitic neoplasm with long latency, and Mo-MuLV induced T-cell leukemia, characterized by an enlargement of the spleen and/or the thymus [3]. The clinical importance of retroviruses as the causative agent of severe diseases in humans and animals, has led to intensive research aimed at developing specific antiretroviral drugs. One basis for the development of new drugs is an increased understanding of the virus life cycle and the enzymes involved in its replication. We showed that topoisomerase-I (topo-I) activity is associated with retroviral particles (Human immunodeficiency virus (HIV), equine infectious anemia virus (EIAV) and Mo-MuLV) and is inhibited by camptothecin (CPT), a specific inhibitor of eukaryotic topo-I [4]. We have also demonstrated that non-cytotoxic doses of CPT inhibited retroviral replication (HIV, EIAV) in acute and chronically infected cells [5,6], and that CPT blocked and prevented retroviral-induced diseases in mice [7].

Topoisomerases are essential enzymes which control and modify the topological state of DNA and are the main targets for anticancer drugs [8–10]. Topo-I catalyzes the concerted breakage and rejoining of a single strand of the DNA helix, and passes another strand through the transient break before resealing.

The breakage step is characterized by a covalent bond formed between the tyrosine residue (present in the enzyme active site) and the phosphate moiety of the DNA backbone. Since this activity resembles tyrosine phosphorylation, we assumed that inhibitors of tyrosine kinases may suppress topo-I activity. It was found that genistein, which blocks the epidermal growth factor (EGF) receptor kinase, also inhibited topo II activity [11]. Our preliminary results indicate that tyrphostins - tyrosine kinase antagonists, inhibit topo-I activity (manuscript in preparation).

Tyrphostins are synthetic compounds which inhibit tyrosine kinases, i.e. EGF [12,13], PDGF receptors [14,15] and the erb-B2/neu kinase [16]. Certain tyrphostins block cell proliferation and their cytotoxicity in tissue culture cells (normal keratinocytes, lymphocytes) is very low and occurs at concentrations above 200 μM [13,16–18].

Since we showed that topo-I inhibitors can act as antiretroviral drugs and that tyrphostin derivatives inhibit eukaryotic topo-I activity in vitro (manuscript in prepa-

* Corresponding author.

SSDI 0014-5793(94)00142-I

ration), we examined the effect of tyrphostins on Mo-MuLV replication, and found that non-cytotoxic doses of two tyrphostin derivatives (AG-555 and AG-18), inhibited Mo-MuLV replication in acute and in chronically infected NIH/3T3 cells.

2. Materials and methods

2.1. Cells and viruses

Cells: NIH/3T3 and NIH/3T3/Mo-MuLV cell lines were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 1% penicillin-streptomycin and 1% L-glutamine.

Mo-MuLV: The culture supernatant of NIH/3T3/Mo-MuLV chronically infected cells (10^6/ml) was collected and centrifuged (3000 rpm, 10 min) from cell debris. 1 ml of this supernatant contained 3×10^6 plaque forming units (PFU).

2.2. Compounds

Tyrophostin derivatives: AG-555, AG-18 and AG-213 were received from Dr. A. Levitzki, Hebrew University, Jerusalem. Stock solutions of the drugs, 10 mM or 100 mM in 100% dimethyl sulfoxide (DMSO), were stored at −20°C and diluted with culture medium before being added to the cells. The final concentration of DMSO in the culture medium was less than 1.5%.

2.3. Mo-MuLV acute infection of NIH/3T3 cells and tyrphostin treatment

Culture supernatant (0.1 ml) containing 3×10^5 PFU of Mo-MuLV was used for infecting 10^5 NIH/3T3 cells treated with polybrene (8 μg/ml) in the presence or absence of different concentrations of AG-555, AG-18 or AG-213, and control cells received equivalent volumes of DMSO. Cells were incubated for 1 h at 37°C, washed and resuspended in fresh media in the presence or absence of tyrphostins (50–150 μM) for 20 h at 37°C.

Reverse transcriptase (RT) activity in the culture media was measured 7 days after infection as described [19], and the IC_{50} (50% inhibiting concentration) for each tyrphostin was determined.

2.4. Tyrphostin treatment of chronically infected cells

NIH/3T3/Mo-MuLV chronically infected cells (10^5/flask) were treated with 50–150 μM AG-555 or AG-18 for 20 h at 37°C. RT activity was measured in the culture medium 48 h following treatment. For long-term tyrphostin treatment, 10^6 NIH/3T3/Mo-MuLV cells were treated with 0.5–10 μM AG-555 or AG-18. Fresh medium containing tyrphostins was added every 4 days, and cells were diluted to 10^6 cells per plate. RT activity was measured in Mo-MuLV particles isolated from the culture medium 13 days after the first tyrphostin treatment. Cell viability was determined by the trypan blue exclusion assay and cell growth by cell counting.

2.4. Tyrphostin cytotoxicity

To determine the cytotoxic effect of tyrphostin derivatives (AG-555, AG-18 or AG-213), NIH/3T3 uninfected cells and NIH/3T3/Mo-MuLV chronically infected cells were cultivated at 10^5 cells/well in 24-well Costar dishes. Following over-night incubation, fresh media containing increasing drug concentrations were added for 20 h. Equivalent volumes of DMSO were administered to untreated control cells. Following incubation all cells were washed and resuspended in fresh medium.

Cell viability was measured by 'Neutral red assay' [20] 48 h after tyrphostin treatment, and the CC_{50} value (cytotoxicity concentration 50%) for each drug was determined.

2.6. Immunoblot analysis

Viral particles from the different treatments were obtained by precipitation of the virus from the culture media as described [5]. The virus pellet was resuspended in a 50 μl RIPA buffer (50 mM Tris-HCl, pH 7.5, 0.5 M $NaHPO_4$, 0.5% NP-40, 0.25% Na-deoxycholate, 0.1% SDS). Equal volumes of viral lysate were analyzed on a 5–20% gradient SDS-PAGE, and Western blot analysis was performed using 1:1500 dilution of Mo-MuLV p15 antibodies. The blot was developed by using the Western blotting detection reagents; enhanced chemiluminescence (ECL) (Amersham).

3. Results

3.1. Tyrphostin derivatives (AG-555, AG-18 and AG-213) inhibit Mo-MuLV replication in acute infection of NIH/3T3 cells

To determine the appropriate concentrations of AG-555, AG-18 and AG-213 which permit viability and growth of NIH/3T3 cells, and NIH/3T3/MoMuLV infected cells, we treated these cells with different doses of the tyrphostin derivatives, and measured cell viability and growth by the Neutral Red Assay [20]. CC_{50} of all three derivatives was observed at concentrations above 200 μM for both cell types (Table 1).

To examine the effect of tyrphostins on Mo-MuLV replication, NIH/3T3 cells were infected with Mo-MuLV (as described in section 2) in the absence or presence of non-cytotoxic doses of tyrphostin derivatives. The cells were treated with different doses of tyrphostins for 20 h, washed and fresh medium was added. Reverse transcriptase activity in the culture media was measured 7 days after infection. Two of the tyrphostin derivatives significantly inhibited Mo-MuLV replication at concentrations 6- to 20-fold lower than the doses required for cytotoxic effect. As can be seen from Table 1, the IC_{50} for AG-55 and AG-18 was 10 μM and 40 μM while the CC_{50} was 210 μM and 250 μM, respectively.

It should be noted that the doses of AG-555 which inhibited Mo-MuLV replication were four times lower than the inhibitory doses of AG-18. Interestingly, AG-213, another tyrphostin derivative, did not affect Mo-MuLV replication (Table 1).

The reduction in Mo-MuLV production following tyrphostin treatment was confirmed by analyzing the presence of virus antigen in the culture media. Virus particles were pelleted, lysed and their proteins were separated by electrophoresis and assayed by Western blot analysis using Mo-MuLV p15 antibodies. p15 was present in the

Table 1
Comparative effect of tyrphostin derivatives on Mo-MuLV replication and cell growth

Compounds	RT inhibition	Cell growth inhibition	
	IC_{50} (μM)	CC_{50} (μM) NIH/3T3	NIH/3T3/ Mo-MuLV
AG-555	10.8 ± 2.1	210 ± 25.4	210 ± 29.1
AG-18	40 ± 10.3	250 ± 23.8	240 ± 14.7
AG-213	No effect	230 ± 18.5	235 ± 31.4

RT inhibition was measured in acute infection of NIH/3T3 cells by Mo-MuLV and cell viability was determined using the 'Neutral red assay' as detailed in section 2. All data are mean ± S.D. from 3 experiments.

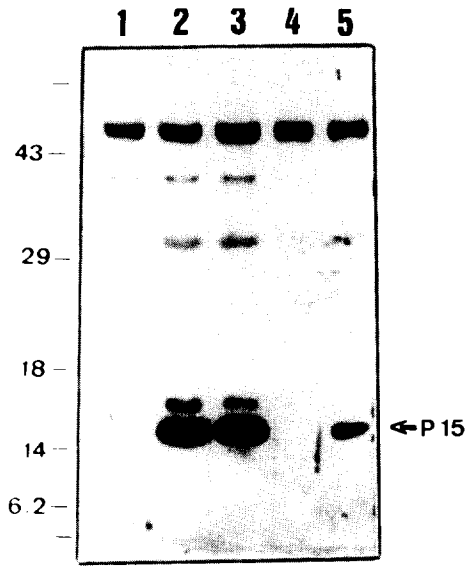

Fig. 1. Effect of tyrphostin AG-555 on Mo-MuLV p15 expression detected by immunoblot analysis. NIH/3T3 cells (10^5 per dish) were infected with Mo-MuLV and treated with AG-555 (100 μM and 10 μM) for 20 h. The virus was pelleted from culture supernatants 7 days after infection. Viral lysate was prepared and the proteins were separated on polyacrylamide gel. Western blot analysis was performed using Mo-MuLV p-15 polyclonal antibodies, and the blot was developed using the ECL immuno-detection kit. The pellet from uninfected and untreated cells (lane 1), virus from untreated infected cells (lane 2), virus from infected cells treated only with 4% DMSO (lane 3) and virus from infected cells treated with 100 μM and 10 μM AG-555 lanes 4 and 5, respectively.

culture media 7 days after infection of NIH/3T3 cells with Mo-MuLV (Fig. 1, lane 2), while the level of p15 was significantly decreased or absent following tyrphostin AG-555 treatment (Fig. 1, lanes 4,5). These results are compatible with the RT results and indicate that tyrphostins added during viral infection prevent subsequent Mo-MuLV replication. This also suggests that the inhibitory effect of AG-555 and AG-18 on Mo-MuLV replication is due to their effect on the virus replication, and is not a result of their antiproliferative property. To examine the inhibitory efficacy of tyrphostins administered following virus infection, NIH/3T3 cells were infected with Mo-MuLV and treated with different doses of tyrphostins one or three days post virus infection. As can be seen in Table 2, 81–86% inhibition of Mo-MuLV replication was observed when 100 μM of tyrphostins were administered one or three days post virus infection. Lower concentrations of these drugs were less effective and at 50 μM, the efficacy of AG-555 was 4-fold higher than AG-18.

3.2. *The effect of AG-555 and AG-18 on Mo-MuLV replication in NIH/3T3/Mo-MuLV chronically infected cells*

Our results indicate that tyrphostin treatment inhibits Mo-MuLV replication in acute infection of NIH/3T3 cells. In order to evaluate their effect on chronically infected cells, NIH/3T3/Mo-MuLV cells were treated with 50 μM or 150 μM of AG-555 or AG-18 for 20 h. Forty-eight hours later, RT activity was measured in the culture medium. As shown in Fig. 2, AG-555 at concentrations of 50 μM and 150 μM inhibited virus replication by 78% to 87%, while equal doses of AG-18 showed a reduction from 33% to 46%. These results indicate that AG-555 significantly reduced Mo-MuLV replication in chronically infected cells and, in this respect, it is more effective than AG-18. We also examined the influence of a long-term treatment of tyrphostins using very low doses of these drugs (0.5–10 μM). Chronically infected 10^6 Mo-MuLV/NIH/3T3 cells were treated with tyrphostins, fresh medium containing these compounds was added every 4 days, and RT activity was measured 13 days after the first tyrphostin treatment. As shown in Fig. 3, 80–90% inhibition of Mo-MuLV replication was observed 13 days after continuous AG-555 treatment. During this period of tyrphostin treatment no effect on cell viability or cell growth was observed. The results also show that treatment with AG-555 was significantly more efficient than with AG-18.

4. Discussion

We have previously shown that the topo I specific antagonist inhibits retroviral replication in vitro [5,6] and prevents the onset of retroviral induced diseases in vivo [7]. In our present work, we demonstrated the ability of tyrphostins, which are tyrosine kinases antagonists, to inhibit Mo-MuLV replication in acute and in chronically infected NIH/3T3 cells. This inhibitory effect is not due to possible cytotoxic effects of the tyrphostins, since non-cytotoxic doses of three different tyrphostin derivatives were used. Moreover, these compounds are

Table 2
Effect of tyrphostins administered after the infection process on Mo-MuLV replication in NIH/3T3 cells

	1 Day post-inf.		3 Days post-inf.	
	RT (cpm)	% Inhibition	RT (cpm)	% Inhibition
Control	19158 ± 390		19158 ± 390	
AG-555 50 μM	4000 ± 220	79 ± 5	6064 ± 178	68 ± 7
AG-555 100 μM	2683 ± 53	86 ± 3	3684 ± 35	81 ± 9
AG-18 50 μM	8317 ± 66	56 ± 3	20446 ± 450	0
AG-18 100 μM	4149 ± 113	78 ± 6	8899 ± 420	53 ± 5

NIH/3T3 cells (10^5) were infected with Mo-MuLV and treated with 50 and 100 μM AG-555 or AG-18 for 20 h. The drugs were added to the culture media 1 or 3 days after virus infection. The data are mean ± S.D. from 3 different experiments.

effective in inhibiting Mo-MuLV replication even when administered three days post virus infection. In addition, we showed that these compounds also reduced Mo-MuLV replication in chronically infected NIH/3T3 cells.

Although a short-term treatment (20 h) with 50 μM–150 μM was effective, a long-term treatment (13 days), using low doses of these drugs (0.5 μM–10 μM), showed a high efficacy in inhibiting Mo-MuLV replication.

Agents such as RT inhibitors [22] which block retroviral replication, act at early stages of the viral infection process, while other agents, such as α-interferon, have been reported to suppress retroviral expression in chronically infected cells [23]. Tyrphostins appear to act as antiviral drugs at both levels and, under certain conditions, are capable of both blocking the initial stage of retroviral infection and reducing viral production in chronically infected cells thus, presenting potential advantages over drugs that function only on one level. The mechanism by which these drugs act as antiviral inhibitors is not clear. Since we found that tyrphostins inhibited topo-I activity in vitro (unpublished data) and previously demonstrated that topo-I specific inhibitors block retrovirus replication [5,6], it is possible that the influence of these drugs on the virus replication is due to their effect on topo-I activity. However, it is also conceivable that tyrphostins act as antiviral drugs by influencing the activity of other viral proteins. The effect of tyrphostins on retrovirus replication may also be due to their impact on cellular proteins involved in the replication of retroviruses (such as, cellular proteins required for efficient virus integration, cellular enzymes which are necessary for the transcription of the viral genome, etc.). It is feasible that some tyrphostins have more than one mechanism of action and AG-555 and AG-18 may affect virus proteins and cellular proteins. For example, tyrphostin AG-213 which is an EGFR kinase blocker, has been found to possess an antiproliferative effect that may not be related to its EGFR kinase inhibitory activity [21].

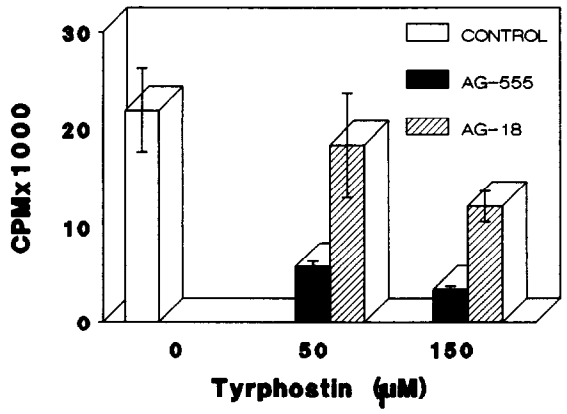

Fig. 2. Effect of tyrophostins on chronically infected NIH/3T3/Mo-MuLV cells. NIH3T3/Mo-MuLV chronically infected cells (10^5 per dish) were treated with 50 μM and 150 μM AG-555 or AG-18 for 20 h, and RT activity in the culture medium was measured 48 hrs post-treatment. The data are mean ± S.D. from 3 different experiments.

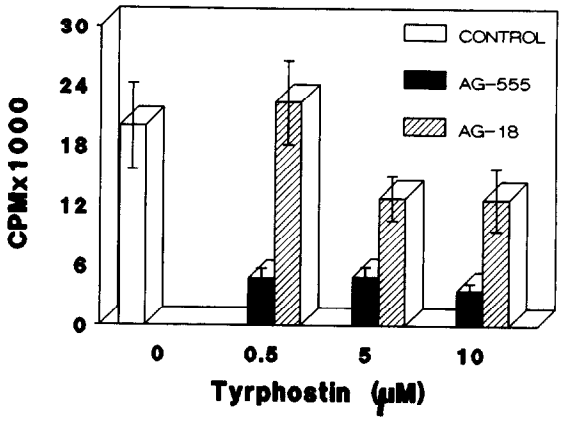

Fig. 3. Long-term treatment of tyrphostin in chronically infected NIH3T3/Mo-MuLV cells. NIH/3T3/Mo-MuLV chronically infected cells (10^6 per dish) were treated with low doses (0.5, 5, 10 μM) of AG-555 or AG-18. Fresh medium containing tyrphostins was added every 4 days, cells were diluted to 10^6 cells per plate, and RT activity was measured 13 days after the first tyrphostin treatment. The data are mean ± S.D. from 3 independent experiments.

Interestingly, we found that AG-555 is significantly more effective in inhibiting Mo-MuLV replication than AG-18, and AG-213 does not have a significant effect. These differences in the retrovirus inhibition are not directly correlated to their anti-tyrosine kinase activity.

AG-555 and AG-213 are very potent protein tyrosine kinase inhibitors, whereas AG-18 exhibits a lower anti-PTK activity (A. Levitzki, personal communication).

Since tyrphostins have an antiproliferative effect but are not toxic to cell growth and the addition of AG-213 to acute infection of NIH/3T3 cells did not inhibit Mo-MuLV replication, we may therefore assume that the inhibitory effect of AG-555 and AG-18 is not solely due to their antiproliferative effect.

Our results suggest that tyrphostins may represent a new class of drug with an antiretroviral potential. The mechanism by which these agents act as antiviral inhibitors and the stages in the virus life cycle which are influenced by these drugs are presently under investigation.

Acknowledgements: This work was supported by research grants from the Israeli Ministry of Health.

References

[1] Hinuma, Y., Nagata, K. and Misoka, M. (1981) Proc. Natl. Acad. Sci. USA 78, 6476–6480.
[2] Gallo, R.C. et al. (1983) Cancer Res. 43, 3892–3899.
[3] Lee, J.C., Horka, I. and Ihle, J.N. (1981) J. Immunol. 126, 715–723.
[4] Priel, E., Showalter, S.D., Roberts, M., Oroszlan, S., Segal, S., Aboud, M. and Blair, D.G. (1990) EMBO J. 9, 4167–4172.

[5] Priel, E., Showalter, S.D., Roberts, M., Orozlan, S. and Blair, D.G. (1991) J. Virol. 65, 4137:4141.
[6] Priel, E., Showalter, S.D. and Blair, D.G. (1991) AIDS Res. Human Retroviruses 7, 65–72.
[7] Priel, E. et al. (1993) J. Virol. 67, 3624–3629.
[8] Orlica, K. and Franco, R.J. (1988) Biochemistry 27, 2253–2259.
[9] Andoh, T., Ishil, K., Suzuki, Y., Ikegami, Y., Kusunoki, Y., Yakemoto, Y. and Okado, K. (1987) Proc. Natl. Acad. Sci. USA 84, 5565–5569.
[10] Gupta, R.S., Gupta, R., Eng, B., Lock, R.B., Ross, W.E., Hertzberg, R.P., Caranfa, M.J. and Johnson, R.K. (1988) Cancer Res. 48, 6404–6410.
[11] Markovits, J., Linassier, C., Fosse, P., Couprie, J., Pierre, J., Jacquemin-Sabion, A., Saucier, J.M., Le-Pecq, J.B. and Larsen, A.K. (1989) Cancer Res. 49, 5111–5117.
[12] Levitzki, A. (1990) Biochem. Pharmacol. 40, 913–920.
[13] Gazit, A., Yaish, P., Gilon, C. and Levitzki, A. (1989) J. Med. Chem. 32, 2344–2352.
[14] Bilder, G.E., Krawiec, J.A., McVety, K., Gazit, A., Gilon, C., Lyall, R., Zilberstein, A., Levitzki, A., Perrone, M.H. and Schreiber, A.B. (1991) Am. J. Physiol. 260, C721–C730.
[15] Bryckaert, M.C., Eldor, A., Gazit, A., Osherov, N., Gilon, C., Fontenay, M., Levitzki, A. and Tobelem, G. (1992) Exp. Cell. Res. 199, 255–261.
[16] Gazit, A., Osherov, N., Posner, I., Yaish, P., Poradosu, E., Gilon, C. and Levitzki, A. (1991) J. Med. Chem. 34, 1897–1907.
[17] Roifman, C., Millis, G.B., Chin, K., Gazit, A., Gilon, C. and Levitzki, A. (1991) J. Immunol. 146, 2965–2971.
[18] Stanley, J.B., Gorczynski, R., Hung, C.K., Love, J. and Mills, G.B. (1990) J. Immunol. 145, 2189–2198.
[19] Goff, S., Traktman, P. and Baltimore, D. (1981) J. Virol. 38, 239–248.
[20] Finter, N.B. (1969) J. Gen. Virol. 5, 419–423.
[21] Faaland, C.A., Mermelstein, F.H., Hagashi, J. and Laski, J.D. (1991) Mol. Cell. Biol. 11, 2697–2703.
[22] Mitsuya, H., Weinhold, P., Furman, A., Clair, M.H. St., Nusinoff-Lehrman, S., Gallo, R.C., Bolones, D., Barry, D. and Broder, S. (1985) Proc. Natl. Acad. Sci. USA 82, 7096–7100.
[23] Ho, D.D., Rota, T.R., Kaplan, J.C., Hartshorn, K., Andrews, C., Schooley, R. and Kirsch, M. (1985) Lancet 1, 602–604.