Cancer Chemother Pharmacol (1997) 39: 561–565

SHORT COMMUNICATION

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Effect of ifosfamide on intracellular glutathione levels in peripheral blood lymphocytes and its correlation with therapeutic response in patients with advanced ovarian cancer

Received: 16 March 1996 / Accepted: 25 July 1996

Abstract The successful outcome of ovarian cancer therapy with alkylating agents and cisplatin is seriously hampered by the development of acquired drug resistance. An increase in intracellular glutathione (GSH) levels in cancer cells is one of the major mechanisms involved. Depletion of GSH overcomes drug resistance and restores the chemosensitivity of malignant cells. Ifosfamide (IFEX), an alkylating agent, has been demonstrated to decrease intracellular GSH levels in vitro in malignant cell lines and in vivo in peripheral blood lymphocytes (PBL) obtained from patients with cancer. We studied the effect of IFEX on intracellular GSH levels in PBL isolated from patients with advanced ovarian cancer who were receiving chemotherapy. A total of 14 patients received IFEX plus mesna as a continuous infusion (1 g/m^2 per day) for 6 consecutive days and cisplatin (100 mg/m^2) as a 24-h continuous infusion on the 6th day. PBL were isolated prior to the initiation of chemotherapy and on the 3rd and 6th days of IFEX infusion. Intracellular GSH levels were determined by a modification of Tietze's method. IFEX caused a 20% or greater suppression of intracellular GSH levels in nine patients, eight of whom achieved complete remission of their disease. Six patients responded poorly to this chemotherapeutic regimen, five of whom showed no significant suppression of GSH levels. These data suggest that IFEX suppresses intracellular GSH levels in PBL from patients with ovarian cancer and that this suppression correlates closely with the subsequent clinical outcome.

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Key words Ifosfamide · Glutathione · Ovarian cancer · Drug resistance

Abbreviations *GSH* Glutathione · *PBL* peripheral blood lymphocytes · *IFEX* ifosfamide

Introduction

Ovarian cancer is the second most common gynaecologic malignancy in females and is the most common cause of gynaecologic cancer deaths. Use of combination of anticancer drugs, mostly cyclophosphamide and cisplatin, is considered the standard therapy for patients who present with stage III–IV disease. Despite the high initial response rates to chemotherapy, cure is achieved in only a few patients [3, 6], primarily due to the development of resistance to the cytotoxic drugs. The pathogenesis of this resistance is multifactorial and the underlying mechanisms vary with the agents used. There is considerable evidence to suggest that the development of resistance to alkylating agents and, possibly, cisplatin is associated with increased intracellular glutathione (GSH) levels [11, 28, 35]. Hence, GSH depletion may be advantageous in the management of these patients.

Drug-resistant tumor cells have been shown to contain levels of GSH several orders of magnitude higher than those measured in wild-type cells. GSH may reduce cytotoxicity by facilitating the metabolism of drugs to less active compounds or by detoxification of the free radicals [2, 25]. Additionally, GSH may enhance the repair of drug-induced injury, primarily at the DNA level. There is also considerable evidence that sensitivity to alkylating agents can be restored by depletion of intracellular GSH [1, 5, 10, 12, 34].

Buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, has been demonstrated to lower GSH levels in human ovarian cancer cell lines, resulting in an

increase in melphalan and carboplatin cytotoxicity [2, 22, 31]. Similarly, in animal studies, BSO decreases GSH levels in the tumor cells, resulting in increased melphalan cytotoxicity and improved survival [27]. Hence, GSH is involved in the development of drug resistance, and its inhibition may restore or enhance the cytotoxic activity of several drugs.

Ifosfamide (IFEX) is an analog of cyclophosphamide. Although it was initially discovered in the early 1970s, severe urotoxicity prevented its widespread use. With the discovery of mesna (sodium 2-mercaptoethanesulfonate) as a uroprotective agent, this drug is being increasingly used in a wide variety of cancers [4, 7, 37]. IFEX is only partially cross-resistant with cyclophosphamide, and response rates of 15–30% have been reported in cyclophosphamide-resistant cases [30, 33]. More recently, IFEX has been demonstrated to reduce GSH levels [21]. This suggests that IFEX may play a significant role in the management of ovarian cancer.

For further elucidation of the effect of IFEX on intracellular GSH levels, 14 patients with histologically proven ovarian cancer were treated with IFEX-cisplatin combination chemotherapy. We studied the effect of IFEX on intracellular GSH levels and correlated this with the subsequent response to chemotherapy.

Patients and methods

Selection of patients

Patients with histologically proven advanced ovarian cancer were eligible for this trial. Only patients whose disease was measurable either clinically, radiologically, or serologically were included in this study. Patients were expected to have a WHO performance status of 0–2, a life expectancy of >3 months, no prior radiation, a WBC count of $>4{,}000/mm^3$, a platelet count of $>100{,}000/mm^3$, a serum creatinine level of <2.0 mg/dl, liver-function tests of <2 times the normal values, and no other malignancy. Patients with prior chemotherapy exposure were also eligible for the trial. Informed consent was obtained in all cases.

Chemotherapeutic regimen

The combination chemotherapy protocol consisted of IFEX at 1 g/m^2 given as a 24-h continuous infusion daily for 6 consecutive days. Similar amounts of mesna were added to the same infusion bag and given for 6 days along with IFEX and for 12 h afterward. Cisplatin at 100 mg/m^2 was given as a 24-h continuous infusion overlapping the 6th day of IFEX infusion. Patients were extensively hydrated prior to, during, and after cisplatin infusion. An institutional protocol to prevent cisplatin-induced nausea and vomiting was followed. This included use of metoclopramide, clemastine, and dexamethasone. No other drug was used.

Response to therapy

The response to combination chemotherapy was determined clinically, serologically, and radiologically. Patients who had clinical disappearance of their disease, normalization of the serological marker CA-125, and the absence of radiologically evident disease on the use of the IFEX-cisplatin combination were considered good responders. Those who failed to meet these criteria were considered poor responders. Drug toxicity was defined according to well-established criteria [26].

Determination of GSH levels

GSH levels were determined in peripheral blood lymphocytes (PBL) isolated from blood samples (5–10 ml) obtained from the patients at the following time points: before the start of chemotherapy, on the 3rd day of IFEX infusion, and at the beginning of the 6th day of infusion immediately prior to the administration of cisplatin. Blood was collected in heparinized tubes and the lymphocytes were isolated using Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.). Cells were washed with phosphate-buffered saline (PBS), counted, and then pelleted down by centrifugation. The pellet was extracted in 0.5 ml of 1 *M* perchloric acid for 1 h. After centrifugation, 0.3 ml of supernatant solution was neutralized with 0.18 ml of ice-cold KOH/MOPS buffer (pH 8.6) and assayed for GSH by a modification of the procedure described by Tietze [15, 36]. GSH content was expressed in nanomoles per 10^6 cells.

Results

This study was carried out on 14 patients; their clinical characteristics are provided in Table 1. The mean age of the patients was 50.4 years (range 33–60 years). The most common histologic subtype encountered was serous cystadenocarcinoma. Five patients presented with International Federation of Gynaecology and Obstetrics (FIGO) stage IV disease. None of them had undergone surgery, and chemotherapy was the only modality offered to these patients. Nine patients had stage III disease. They all underwent debulking surgery. Post operatively, five of them had bulky residual disease (>1.5 cm). The other four patients had minimal residual disease (<1.5 cm) postoperatively. However, they continued to show elevated levels of the tumor marker CA-125. Most of the patients were chemotherapy-naive. Three had received previous chemotherapy, cisplatin and cyclophosphamide in all cases, and had relapsed. GSH levels were determined prior to the institution of chemotherapy and on the 3rd and 6th days of IFEX infusion. The values are provided in Table 1. Nine patients showed a 20% or greater reduction in GSH levels during IFEX infusion. Eight of these nine patients achieved complete remission of their disease and were considered good responders as defined above. Six patients responded poorly to this chemotherapeutic regimen. Five of them showed no significant fall in GSH levels. The only exception was a patient in whom a decline in GSH levels was observed on the 6th day, however, the value was above the baseline levels on the 3rd day. The effect of IFEX on GSH levels in PBL of patients showing a good response is presented in Fig. 1.

Table 1 Clinical characteristics and changes in the intracellular GSH values found in PBL of the study patients and their correlation with the response to IFEX-cisplatin combination chemotherapy

Patient number	Age (years)	Histologic subtype	Extent of disease (stage)	Previous chemotherapy	GSH levels (nmol/10^6 cells)				Response to therapy
					Baseline[a]	Day 3	Day 6	Maximal % decline[b]	
1	60	Serous	III (minimal)	None	0.74	0.25	0.05	93.2	Good
2	56	Endometrioid	III (bulky)	None	0.55	0.32	0.25	54.5	Good
3	64	Serous	III (bulky)	None	0.34	0.03	0.09	91.2	Good
4	59	Mucinous	III (minimal)	None	0.51	0.38	0.67	25.5	Good
5	49	Mucinous	IV	None	0.83	0.54	0.17	79.5	Good
6	40	Undifferentiated	III (bulky)	None	0.48	0.09	0.21	81.3	Good
7	42	Serous	III (minimal)	None	0.5	0.19	0.28	62	Good
8	50	Serous	IV	Cisplatin-cyclo-phosphamide	2.11	0.61	1.34	71	Good
9	56	Undifferentiated	III (minimal)	None	0.80	0.96	1.61	–	Poor
10	52	Serous	IV	None	0.22	0.21	0.2	9.1	Poor
11	33	Serous	III (bulky)	None	0.43	0.75	0.45	–	Poor
12	46	Serous	IV	None	0.25	0.36	0.55	–	Poor
13	51	Undifferentiated	III (bulky)	Cisplatin-cyclo-phosphamide	0.39	0.33	0.83	15.4	Poor
14	58	Serous	IV	Cisplatin-cyclo-phosphamide	0.52	0.55	0.41	21.2	Poor

[a] Mean values $\pm$ SD recorded for good responders and poor responders were 0.757 ± 0.57 and 0.435 ± 0.21 nmol/10^6 cells, respectively
[b] Mean values $\pm$ SD recorded for good responders and poor responders were $69.8 \pm 22.3\%$ and $7.6 \pm 2.8\%$, respectively

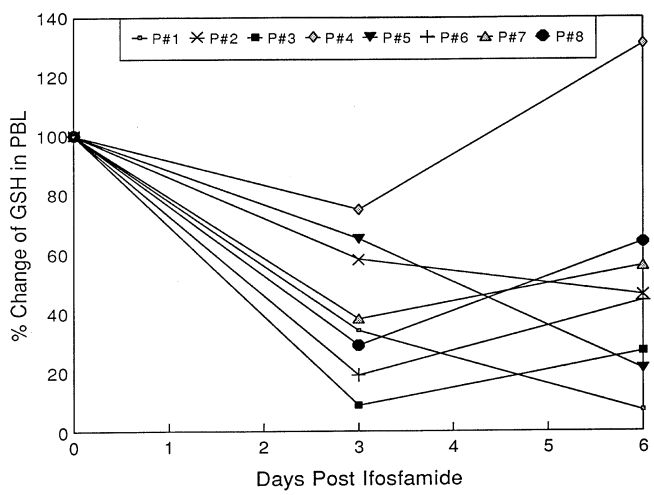

Fig. 1 Percentage of change in GSH levels in PBL of patients who responded well to the treatment (*P* Patient)

It was interesting that the mean ($\pm$ SD) baseline GSH levels measured among good responders (0.757 ± 0.568 nmol/10^6 cells) and poor responders (0.435 ± 0.21 nmol/10^6 cells) were found not to be statistically significantly different as analyzed by Student's *t*-test for equality of means. However, the mean percentage of decline in GSH levels noted after IFEX treatment among good responders (69.8%) and poor responders (7.6%) revealed a statistically significant difference at $P < 0.005$ when analyzed by the same test.

Discussion

Lind et al. [21] demonstrated the ability of IFEX and its metabolites to lower intracellular GSH levels in vitro and in vivo. In a single patient receiving an 8-h infusion of IFEX, a marked decrease in GSH levels to about 30% of the original value was observed. The significance of this observation, however, remains undetermined. A similar effect in vivo was observed by Meier et al. [24]. It has been suggested that this effect may be partly mediated by inhibition of plasma GSH peroxidase activity by IFEX [29]. We observed a similar inhibitory effect of IFEX on intracellular GSH levels in PBL obtained from patients with ovarian carcinoma. We also observed a close association of this inhibitory effect with the subsequent response to chemotherapy. In all, 9 of the 14 patients showed a 20% or greater decline in GSH levels during IFEX infusion; all but 1 achieved complete remission of their disease. In the patient who did not respond despite the observation of a 20% reduction in GSH levels on day 6, GSH levels were above baseline value on day 3 of IFEX infusion. This transient decline in GSH levels may not have been reflective of the overall effect. Five patients had less than a 20% decline in GSH levels. All of them responded poorly to the combination chemotherapy. These results suggest that the decline in GSH levels induced by IFEX closely correlates with the therapeutic response.

There could be several reasons for the observed effects. A fall in GSH levels may be due to the direct effect

of IFEX on GSH metabolism [29]. This reduction in GSH levels may prevent the subsequent development of resistance to IFEX and, hence, correlates with the therapeutic response. Another possibility would involve the increased metabolic conversion of IFEX into 4-OH-IFEX in responders. An additional explanation might be sensitization of the tumor cells to cisplatin cytotoxicity due to decreased GSH levels. In the present study, cisplatin was given on the 6th day of IFEX infusion. Decreased GSH levels observed at that time may also have allowed enhanced cisplatin-induced cytotoxicity [8, 9, 13, 14, 23]. It is, however, unclear whether GSH is a major determinant of cisplatin cytotoxicity [1, 9]. Because we used both IFEX and cisplatin, our study does not provide an adequate explanation. Administration of IFEX as a single agent and correlation of its effect on GSH levels with the therapeutic response may help to elucidate further the underlying mechanism.

It must be emphasized that in our study, GSH levels were determined in PBL. Other investigators have applied similar methods to determine GSH levels in vivo [21, 24]. It is unclear whether similar effect is observed on the tumor cells. Since data are not available on this subject, we cannot draw any definitive conclusion.

The chemotherapeutic regimen used in this study also included intravenous mesna. Mesna was mixed with IFEX in the same bag and at the same dose and was given for an additional 12 h after the IFEX infusion. Mesna may influence the GSH status of the cells [16, 17]. Stimulation of GSH synthesis and an increase in GSH content has been described in Chinese hamster ovarian cells. Two recent studies, however, demonstrate that mesna has no influence on the GSH levels in PBL [24, 32]. Due to these data and to the presence of mesna throughout the IFEX infusion, it appears unlikely that mesna had any significant effect on the GSH levels observed in our patients.

Another important issue is the effect of depletion of GSH on normal cellular functions. Depletion of GSH can result in cellular dysfunction. In PBL, depletion of GSH has been demonstrated to decrease the proliferative activity of the cells [18]. There is significant inhibition of [^{3}H]-thymidine incorporation in interleukin-2-expanded PBL. Similarly, suppression of GSH peroxidase activity may facilitate the peroxidation of membrane lipids, resulting in enhanced toxicity. GSH depletion with resultant loss of an important cellular protective mechanism against toxic agents and drugs may make cells more vulnerable to drug toxicity. Hence, the frequency or degree of IFEX-induced side effects may increase. In our ongoing study we have not yet observed any unexpected increase in the incidence or severity of side effects. However, further trials are necessary to exclude this possibility completely.

Information regarding the GSH-inhibitory effect of IFEX may be important in the formulation of drug combinations. Combination of IFEX with other drugs that have GSH-dependent metabolism may help to enhance their cytotoxic activity by reducing GSH levels. Conversely, taxol cytotoxicity is decreased by GSH depletion and, hence, should be used cautiously with IFEX [20]. In vitro activity and interactions of taxol and IFEX support this hypothesis [19].

In conclusion, we observed substantial inhibition of intracellular GSH levels in PBL by IFEX. We also observed a close relationship between GSH inhibition and the response to cytotoxic therapy. A prospective trial is in progress to evaluate the efficacy and toxicity of IFEX and cisplatin in previously untreated patients with advanced ovarian cancer.

Acknowledgements We gratefully acknowledge the help rendered by Dr. Zubair K. Khan and Mehreen Sabih of the National Cancer Institute and by Messers Jawad Kirmani and Shiraz Farooq of the Aga Khan University in providing the clinical samples. The authors wish to thank Mr. Sulaiman Gilani for his help in preparing the manuscript. This work was supported in part by a grant to Dr. M. Perwaiz Iqbal from the University Grants Commission, Islamabad, Pakistan.

References

1. Andrews PA, Murphy MP, Howell SB (1985) Differential potentiation of alkyating and platinating agent cytotoxicity in human ovarian carcinoma cells by glutathione depletion Cancer Res 45:6250
2. Arrick BA, Nathan CF (1984) Glutathione metabolism as a determinant of therapeutic efficacy: a review. Cancer Res 44:4224
3. Averette HE, Donato DM (1990) Ovarian carcinoma: advances in diagnosis, staging and treatment. Cancer 65:703
4. Brade W, Seeber S, Herdrich K (1986) Experimental and clinical activity of ifosfamide and cyclophosphamide. Cancer Chemother Pharmacol 18 [Suppl 2]:S1
5. Canada A, Herman L, Kidd K, Robertson C, Trump D (1993) Glutathione depletion increases the cytotoxicity of melphalan to PC-3, an androgen-insensitive prostate cancer cell line. Cancer Chemother Pharmacol 32:73
6. Chabner BA, Fine RL, Allegra CJ, et al. (1984) Cancer chemotherapy: progress and expectations. Cancer 54:2599
7. Dechant KL, Brogden RN, Pilkington T, Faulds D (1991) Ifosfamide/mesna. A review of its antineoplastic activity, pharmacokinetic properties and therapeutic efficacy in cancer. Drugs 42:428
8. Edwards PG (1988) Evidence that glutathione may determine the differential cell-cycle phase toxicity of a platinum (IV) antitumor agent. J Natl Cancer Inst 80:734
9. Eichholtz-Wirth H, Reidel G, Hietel B (1993) Radiation-induced transient cisplatin resistance in murine fibrosarcoma cells associated with elevated metallothionein content. Br J Cancer 67:1001
10. Green JA, Vistica DT, Young RC, Hamilton TC, Rogan AM, Ozols RF (1984) Potentiation of melphalan cytotoxicity in human ovarian cancer cell lines by glutathione depletion. Cancer Res 44:5427
11. Gurtoo HL, Hipkens JH, Sharma CD (1981) Role of glutathione in the metabolism dependent toxicity and chemotherapy of cyclophosphamide. Cancer Res 41:3584
12. Hamilton TC, Winkler C, Louie K, Batist G, Behrens B, Ozols RF (1985) Augmentation of adriamycin, melphalan, and

cisplatin cytotoxicity in drug-resistant and sensitive human ovarian carcinoma cell lines by glutathione depletion. Biochem Pharmacol 34 : 2583
13. Hamilton TC, Lai GM, Rothenberg ML, Fojo A, Young RC, Ozols RF (1989) Mechanisms of resistance to alkylating agents and cisplatin. In: Ozols RF (ed) Cancer treatment and research: drug resistance. Martinis Nijhoff, Boston, p 151
14. Hospers GAP, Mulder NH, De Jon B, De Ley L, Uges DRA, Fichtinger-Schepmann AMJ, Scheper RT, DeVries EGE (1988) Characterization of a human small cell lung carcinoma cell line with acquired resistance to *cis*-diamminedichloroplatinum(II) in vitro. Cancer Res 48 : 6803
15. Iqbal MP, Malik IA, Mehboobali N, Waqar MA (1993) A spectrophotometric method for glutathione levels in normal and tumor cells. Proceedings, 5th national chemistry conference, Quaid-i-Azam University, Islamabad, Pakistan
16. Issels RD, Nagele A (1989) Promotion of cystine uptake, increase of glutathione biosynthesis, and modulation of glutathione status by *S*-2-(3-aminopropylamino)ethyl phosphorothioic acid (WR-2721) in Chinese hamster cells. Cancer Res 49 : 2082
17. Issels RD, Nagele A, Eckert KG, et al (1988) Promotion of cystine uptake and its utilization for glutathione biosynthesis induced by cysteamine and *N*-acetylcysteine. Biochem Pharmacol 37 : 881
18. Issels RD, Meier TH, Muller E, Multhoff G, Wilmanns W (1993) Ifosfamide induced stress response in human lymphocytes. Mol Aspects Med 14 : 281
19. Klaassen U, Harstrick A, Vanhoefer U, Schleucher N, Stahl M, Seeber S, Wilke H (1994) Activity and schedule-dependent interactions of taxol, ifosfamide and etoposide in cisplatin sensitive and refractory human ovarian carcinoma cell lines as a basis for second line protocols (abstract 4917). Ann Oncol 5 [Suppl 8] : 98
20. Liebman JE, Hahn SM, Cook JA, Lipshultz C, Mitchell JB, Kaufman DC (1993) Glutathione depletion by L-buthionine sulfoximine antagonizes taxol cytotoxicity. Cancer Res 53 : 2066
21. Lind MJ, McGowan AT, Hadfield JA, Thatcher N, Crowther D, Fox BW (1989) The effect of ifosfamide and its metabolites on intracellular glutathione levels in vitro and in vivo. Biochem Pharmacol 38 : 1835
22. Louie KG, Behrens BC, Kinsella TJ, Hamilton TC, Grotzinger KR, McKoy WM, Winker MA, Young RC, Ozols RF (1985) Radiation survival parameters of antineoplastic drug-sensitive and resistant human ovarian cancer cell lines and their modification by buthionine sulfoximine. Cancer Res 45 : 2110
23. Masuda H, Ozols RF, Lai, GM, et al. (1988) Increased DNA repair as a mechanism of acquired resistance to *cis*-diamminedichloroplatinum(II) in human ovarian cancer cell lines. Cancer Res 48 : 5713
24. Meier T, Allenbacher A, Mueller E, Multhoff G, Botzler C, Weisnet M, Issels R (1994) Ifosfamide induced depletion of glutathione in human peripheral blood lymphocytes and protection by mesna. Anticancer Drugs 5 : 403
25. Meister A (1983) Selective modification of glutathione metabolism. Science 220 : 472
26. Miller AB, Hoogstraten B, Staquet M, Winkler A (1981) Reporting results of cancer treatment. Cancer 47 : 207
27. Ozols RF, Louie KG, Plowman J, et al. (1987) Enhanced melphalan cytotoxicity in human ovarian cancer in vitro and in tumor bearing nude mice by buthionine sulfoximine depletion of glutathione. Biochem Pharmacol 36 : 147
28. Ozols RF, O'Dwyer PJ, Hamilton TC, Young RC (1990) The role of glutathione in drug resistance. Cancer Treat Rev 17 : 45
29. Saito G, Yoshimura S, Iwasaki K, Kuroshima Y, Moriuchi T (1994) Suppression of plasma glutathione peroxidase activity by ifosfamide. Asia Oceania J Obstet Gynaecol 20 : 441
30. Slater AJ, Green JA (1989) Phase II study of cisplatin and ifosfamide in alkylating agent resistant ovarian cancer. Gynaecol Oncol 32 : 233
31. Somfai-Relle S, Suzukake K, Vistica BP, Vistica DT (1984) Reduction in cellular glutathione by buthionine sulfoximine and sensitization of murine tumor cells resistant to L-phenylalanine mustard. Biochem Pharmacol 33 : 485
32. Stofer-Vogel B, Cerny T, Kupfer A, Junker E, Lauterburg BH (1993) Depletion of circulating cyst(e)ine by oral and intravenous mesna. Br J Cancer 68 : 590
33. Sutton GP, Blessing JA, Homesley HD, et al (1989) Phase II trial of ifosfamide and mesna in advanced ovarian carcinoma: a Gynecologic Oncology Group study. J Clin Oncol 7 : 1672
34. Suzukake K, Petro BJ, Vistica DT (1982) Reduction in glutathione content of L-PAM-resistant L1210 cells confers drug sensitivity. Biochem Pharmacol 31 : 121
35. Suzukake K, Petro BJ, Vistica DT (1983) Dechlorination of L-phenylalanine mustard by sensitive and resistant tumor cells and its relationship to intracellular glutathione content. Biochem Pharmacol 32 : 165
36. Tietze F (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Anal Biochem 27 : 502
37. Zalupski M, Baker LH (1988) Review: ifosfamide. J Natl Cancer Inst 8 : 555