Cancer Chemother Pharmacol (1987) 20: 133–136

Cancer Chemotherapy and Pharmacology



# DNA Cross-Linking following exposure to *cis*-platinum in primary and serially passaged cultured cells derived from two murine fibrosarcomas*

**David Murray, Ivan Basic**, Luka Milas, and Raymond E. Meyn**

Department of Experimental Radiotherapy, The University of Texas System Cancer Center, Box 66, M. D. Anderson Hospital and Tumor Institute, 1515 Holcombe Blvd., Houston, TX 77030, USA

**Summary.** We compared the kinetics of the repair of total (ISC plus DPC) cross-links and of proteinase-resistant (ISC) cross-links in cultured cells derived from two murine fibrosarcoma tumors, FSA and NFSA, after treatment with *cis*-platinum (cis-DDP), using a modification of the alkaline elution technique. The two tumors had previously been characterized for their response to *cis*-DDP in vivo; FSA cells gradually removed cross-links from their genome, whereas the NFSA cells showed no capacity to repair these lesions. The aim of the present study was to establish whether treatment of cells from these same two tumors grown under controlled culture conditions would affect either the nature of the lesions induced by *cis*-DDP or the kinetics of repair of these lesions when compared with tumors treated with *cis*-DDP in vivo. The culture conditions represent two situations: in the first, the cells in culture approximated the proportion of tumor and normal host cells present in vivo, and in the second, the normal host cells had been eliminated by subculturing to produce cultures composed entirely of tumor cells. All cells were exposed to *cis*-DDP (either 10 or 20 μg/ml) for 1 h. The relative amounts of total *cis*-DDP-induced DNA cross-links and of ISCs were then determined at various times after treatment. The results show that there was little difference in the behavior of these cultured cells compared to the in vivo response of the tumor from which they were derived. For FSA, each cell culture exhibited a capacity to repair DNA cross-links comparable to that of the tumor in vivo. For NFSA, the passaged cells again paralleled the behavior of that tumor in vivo, although in this case by showing no measurable capacity to repair cross-links. The absence of a significant repair response in the NFSA tumor therefore appears to be an intrinsic characteristic of these tumor cells.

## Introduction

In a recent study [11], we examined the DNA-damaging effects of *cis*-DDP in mice, and we showed that cells from a variety of normal tissues exhibited no capacity to repair *cis*-DDP-induced ISCs or DPCs within 24 h after treatment. We also compared the kinetics of the repair of ISCs and DPCs in two mouse fibrosarcomas, FSA and NFSA, after treatment with *cis*-DDP in vivo [11]. The two tumors exhibited a very different pattern of response to *cis*-DDP. While NFSA cells showed no capacity to repair these lesions, the FSA cells more closely resembled cultured cell lines in that they gradually removed cross-links from their genome. Various intrinsic and environmental factors could contribute to this difference between the tumors, such as the extent of their infiltration by normal host cells or contamination with stromal cells. The NFSA tumor contains a remarkably high proportion of normal host cells, approximately 80% of the cells being macrophages, whereas the FSA tumor contains a much lower proportion (20%–30%) of macrophages [6, 10]. Since our previous studies [11] have shown that normal tissues, including lymphoid and bone marrow, could not repair such DNA cross-links, one possible explanation for the lack of a repair response in *cis*-DDP-treated NFSA tumors in vivo may therefore be a masking of repair in NFSA tumor cells by this larger component of normal host cells.

The purpose of the present study was to investigate the possible role of host cell content in the response of these two fibrosarcomas in vivo by determining DNA cross-linking at various times after exposure of the cells to *cis*-DDP in vitro. Two different situations were investigated using cell cultures derived from each tumor, one which approximated the proportion of cells present in vivo, and a second in which the host cells were eliminated to produce cultures composed entirely of tumor cells. The behavior of these cells in culture is compared with that observed previously for the tumors treated with *cis*-DDP in vivo [11].

## Materials and methods

*Cell culture methods.* FSA [17] and NFSA [1] tumors were generated by s.c. injection of $5 \times 10^5$ tumor cells into the hind legs of recipient mice. The tumors growing and treated in situ will henceforth be referred to as FSA-0 and NFSA-0. Primary cultures (referred to as FSA-1 and NFSA-1 respectively) were established from these two tumors as described by Basic et al. [2]. Single-cell suspen-

* This investigation was supported by USPHS grant CA-23270 awarded by the National Cancer Institute.

** On leave from Department of Animal Physiology, Faculty of Sciences, The University of Zagreb, Zagreb, Yugoslavia

*Offprint requests to:* David Murray

*Abbreviations:* *cis*-DDP- *cis*-platinum (II); PSA, Puck's saline A; CLF, cross-link factor; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; ISC, DNA-interstrand cross-link; DPC, DNA-protein cross-link

sions from each tumor were prepared as described previously [8]. Cell numbers were counted with a hemacytometer.

Tumor cells ($5 \times 10^6$ FSA-0 and $10^7$ NFSA-0) suspended in 10 ml McCoy's 5A medium (Hsu's modification) supplemented with 20% FBS (both Gibco, Grand Island, NY) were seeded into 10-cm tissue culture dishes. Following overnight incubation at 37° C, the medium was decanted, and the attached cells were washed twice with PSA. At this point, cells were removed by scraping with a rubber policeman and were used to determine the percentage of macrophages using the Fc-receptor technique [6]. The cellular composition of FSA-1 and NFSA-1 cultures closely resembled that of the respective tumors measured in situ [6, 10]; FSA-1 cells contained 27% macrophages and NFSA-1 cells 83% macrophages.

The cells were subcultured by trypsinization (0.025% trypsin) for 5 min at 37° C. Detached cells were washed by centrifugation, and new cultures (referred to as FSA-2 and NFSA-2) were grown for 4–6 days until they reached a density of about $10^7$ cells/dish. The FSA-2 cultures contained no macrophages at this stage. For NFSA, subculturing was continued up to the fourth passage to produce NFSA-4 cells, at which point there was no measurable macrophage population in the culture. FSA-2 and NFSA-4 generations were used in the experiments described below because they represented cell cultures composed entirely of tumor cells.

*Drug treatments. cis*-DDP (Bristol Laboratories, Syracuse, NY) was dissolved in serum-free alpha-minimum essential medium (Gibco, Grand Island, NY) to give a final concentration of either 10 or 20 μg/ml. Before treatment, the growth medium was removed from the cells, followed by two washes with PSA; 10 ml of the appropriate *cis*-DDP solution was then added, and the cells were incubated at 37° C for 1 h. After 1 h, the drug was removed, the cells were washed twice with PSA, 10 ml of growth medium containing 20% FBS was added, and the cultures were returned to the incubator at 37° C for various time periods up to 24 h. After this incubation, the medium was removed and the cells were harvested by scraping with a rubber policeman and worked into a single-cell suspension (in ice-cold PSA containing 5 m*M* EDTA) for the determination of DNA damage.

*Alkaline elution.* The alkaline elution technique originally developed for use with radioactively-labeled cells [5] has been adapted for use with unlabeled cells by using the fluorescent dye Hoechst 33258 to assay the eluted DNA; this method for the determination of *cis*-DDP-induced DNA cross-links, and the calculation of CLFs from the resulting DNA elution profiles, have been described in detail elsewhere [11, 12]. All data are the average (± S. E.) of three or more separate experiments. Previous in vitro [7, 15, 19] and in vivo [11] studies have shown that the rate of elution of DNA from *cis*-DDP-treated cells can be influenced by both ISCs and DPCs. The contribution of the latter lesions to the overall retardation of the DNA elution rate was estimated by proteinase K digestion [12]. Those cross-links that were sensitive to digestion by proteinase K are believed to represent covalent attachment of the DNA to proteins (DPCs), whereas the residual proteinase-resistant lesions are presumed to represent cross-linking of opposite DNA strands (ISCs).

*Tumor regrowth delay assay.* When the tumors reached a mean diameter of 8 mm, the mice (between 6 and 10 per group) were injected i.p. either with saline or with *cis*-DDP (12 mg/kg). Three mutually orthogonal diameters of the tumors were measured at 2-day intervals using a vernier caliper, and the means were calculated. Measurements were made either until the animals died or until the mean tumor diameter reached approximately 20 mm.

## Results

Immediately after treatment with *cis*-DDP (either 10 or 20 μg/ml), the level of cross-linking in each of the four tumor-cell cultures (FSA-1, FSA-2, NFSA-1, and NFSA-4) was relatively low; however, in each case, the level of cross-linking increased gradually with time until a maximum effect was observed at about 6 h after removal of the drug. On the basis of preliminary experiments, cross-links were measured at 6 h and 24 h after treatment, both with and without digestion with proteinase K. The 6-h measurements provide an estimate of the maximum amount of cross-linking reached in each cell culture, while the change in cross-linking that occurs during the 6–24 h period can be taken as an indication of the repair proficiency of the cells. For each of the four tumor-cell lines, the dose responses relating the degree of DNA cross-linking to the concentration of *cis*-DDP, measured at either 6 h or 24 h after treatment, were linear within the *cis*-DDP-dose range studied (data not shown). The slopes of these drug dose dependence curves were determined by linear regression analysis (Table 1). There are several points to note from these data:

First, for the FSA cells, the maximum (i.e., 6-h) levels of both total cross-links and ISCs achieved were similar in FSA-1 and FSA-2. Furthermore, in both FSA-1 and FSA-2 cells the level of total cross-linking at 24 h had decreased significantly compared to that at 6 h. Because these doses of *cis*-DDP produced no detectable DNA strand breaks at either 6 h or 24 h after treatment (data not shown), and because we have previously shown that this effect could not be due to DNA replication during the repair incubation "diluting" the level of DNA lesions [12], these data suggest that 30%–40% of the total DNA cross-links had been repaired during the 6–24 h period in each of the cultures. ISCs accounted for about 30% of the total retardation of the DNA elution rate at either 6 h or 24 h, and these lesions were repaired with similar kinetics to the total cross-linking effect, about 30% also being repaired by 24 h in both FSA-1 and FSA-2.

Second, for NFSA-1 and NFSA-4, the maximum levels of DNA damage achieved were again similar to each other, although somewhat lower than those achieved in the equivalent FSA culture. However, in contrast to the FSA cultures, there was little subsequent change in the CLF between 6 and 24 h, suggesting that few of the DNA cross-links present at 6 h were being repaired. ISCs accounted for about 19% of the total retardation of the DNA elution rate in both NFSA-1 and NFSA-4 cells, and these lesions were not appreciably repaired up to 24 h after treatment.

The response of these tumors treated with *cis*-DDP in vivo, measured using the tumor regrowth delay assay, is

**Table 1.** Slopes of dose-response curves relating the degree of *cis*-DDP-induced DNA cross-linking to the concentration of *cis*-DDP in cultured mouse tumor cells

| Cell type | 6-h CLF | | | 24-h CLF | | | % change in CLF (6 to 24 h) | |
|---|---|---|---|---|---|---|---|---|
| | Total | Pro-K--resistant | % Pro-K--resistant | Total | Pro-K--resistant | % Pro-K--resistant | Total | Pro-K--resistant |
| FSA-0[a] | – | – | 22 | – | – | 20 | −51 | – |
| FSA-1 | 3.33±0.8 | 1.08±0.13 | 32 | 2.36±0.44 | 0.74±0.14 | 31 | −29 | −31 |
| FSA-2 | 4.13±1.12 | 1.06±0.22 | 26 | 2.45±0.43 | 0.76±0.08 | 31 | −41 | −28 |
| NFSA-0[a] | – | – | 19 | – | – | 16 | 0 | – |
| NFSA-1 | 2.43±0.51 | 0.47±0.12 | 19 | 2.49±0.52 | 0.60±0.17 | 24 | +2 | +28 |
| NFSA-4 | 3.23±0.80 | 0.63±0.23 | 20 | 2.94±0.94 | 0.67±0.11 | 23 | −9 | +6 |

Units of slope are CLF per unit 10 μg/ml dose of *cis*-DDP, determined by linear regression analysis
[a] Data from [11]

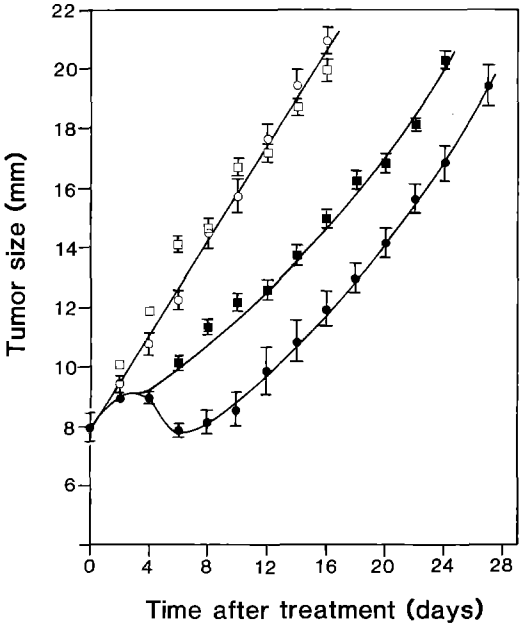


**Fig. 1.** Growth of NFSA-0 (□, ■) and FSA-0 (○, ●) leg tumors in untreated control animals (□, ○) and after administration of *cis*-DDP, 12 mg/kg i.p. (■, ●)

shown in Fig. 1. The growth rates for the untreated tumors were quite similar. Treatment of the animals with *cis*-DDP (12 mg/kg) delayed the growth of both tumors, although the growth of the FSA-0 tumor was delayed to a significantly greater extent than that of the NFSA-0 tumor, paralleling the observation that the NFSA-0 tumor is resistant to a variety of antitumor agents, including cyclophosphamide [9], melphalan (unpublished data), and X-rays [10], compared with FSA-0.

## Discussion

In cultured mammalian cells, *cis*-DDP produces various DNA lesions such as monofunctional adducts, bifunctional binding to a single base moiety, and DNA cross-links of several types (DNA-intrastrand cross-links, ISCs or DPCs) [14, 18]. Although the importance of DNA damage in the cytotoxic action of *cis*-DDP in vitro has frequently been demonstrated, the relative contribution of these lesions to cytotoxicity is not well understood [13, 14, 16]. Cytotoxicity appears to be related both to the initial levels of DNA damage and to the efficiency of repair processes in removing these lesions from the DNA [e.g., see 3, 4, 7]. While it appears that bifunctional adducts may be responsible for cell inactivation, the earlier evidence that ISCs were probably the lethal lesions [19] has been questioned, and a more important role for N7-N7 intratrand cross-links between adjacent guanine residues has been suggested [14]. Although DPCs have been envisaged as relatively unimportant, since *trans*-DDP produces DPCs efficiently but is much less toxic than *cis*-DDP [18], those DPCs produced by *cis*-DDP appear to persist much longer in the cells [13, 15]. Recent in vivo studies using a rat hepatoma indicate that *cis*- and *trans*-DDP selectively cross-link specific proteins, particularly cytokeratins, to DNA [13], suggesting a possible biological relevance for these adducts.

Irrespective of their role in cytotoxicity, these different DNA lesions may provide a useful endpoint for examining the effects at the DNA-damage level of chemotherapy agents, such as *cis*-DDP, that have DNA as their target, and particularly the operation of DNA repair mechanisms in response to these lesions. In this study, we have examined the induction and repair of *cis*-DDP-induced ISCs and DPCs in cell cultures derived from two mouse fibrosarcoma tumors, FSA-0 and NFSA-0, which we previously showed to behave very differently from each other in vivo [11]. Our approach involved comparing the two tumors under controlled culture conditions designed to represent two situations, one in which the culture approximated the proportion of cells present in vivo (FSA-1 and NFSA-1) and a second in which normal host-derived cells, which represent a large proportion of the cells in vivo [6, 10], had been eliminated by subculturing to produce cultures composed almost exclusively of tumor cells (FSA-2 and NFSA-4). By using these systems, the influence of pharmacokinetic, immunological and other host-mediated effects on the response of the cells to *cis*-DDP has been eliminated, while further subculturing of the cells has enabled selective examination of the DNA damage and repair characteristics of the tumor cells independently of the host-cell population.

The most striking observation from these data was that the cultures derived from each of the tumors retained essentially the same DNA damage characteristics exhibited by the tumors after treatment in vivo [11] with respect to both the type (i.e., the ratio of ISCs to DPCs) and rate of

repair of the lesions induced (see Table 1). Furthermore, each of the FSA cell populations treated either in vivo or in vitro had somewhat higher initial levels of both ISCs and DPCs than the equivalent NFSA population. These observations suggest that the processes that control both the formation and repair of such lesions in vivo are retained when the tumor cells are placed in short-term culture. In the case of FSA, this is not too surprising considering that the majority of the cells that make up the volume of the tumor in vivo (FSA-0) are themselves tumor cells. For NFSA, on the other hand, the finding that the temporal characteristics of the DNA cross-linking effect of both NFSA-1 and NFSA-4 cells closely resembled those of the NFSA-0 tumor was more unexpected. In particular, in the case of NFSA-4 cells, for which any contribution from normal host cells had been eliminated, it was somewhat surprising that these cells still showed no measurable capacity to repair any of their ISCs or DPCs. The absence of a measurable repair response to *cis*-DDP-induced DNA cross-links in NFSA-0 therefore appears to be a characteristic of the whole population of cells in the tumor, i.e., both normal and neoplastic cells. This suggests that the ability to repair *cis*-DDP-induced DNA damage is not ubiquitous among tumor cells.

Finally, it is interesting to examine the possible relationship between these measurements of DNA cross-linking and the biological response of these two tumors after treatment with *cis*-DDP in vivo. This analysis is, however, complicated by several factors. First, the cytotoxic effects of *cis*-DDP appear to be related both to the initial levels of DNA damage induced and to the efficiency of repair processes in removing these lesions from the DNA, although there is no clearly defined means of quantitatively relating differences in repair rate and sensitivity [7]. Second, these particular lesions may not be cytotoxic [14] and as such may elicit a different repair response to those lesions that are lethal. Furthermore, a variety of additional factors, such as cell heterogeneity or immunological effects, may play an important role in determining tumor response. These two tumors do, in fact, represent extremes in terms of host-cell infiltration and immunogenicity [6].

FSA-0 cells treated in vivo had somewhat higher initial levels of both ISCs or DPCs than NFSA-0 [11]; however, FSA cells invariably showed a greater capacity to repair these lesions than their NFSA counterparts. On the basis of these two tumors, then, it is difficult to predict the response of tumors to *cis*-DDP from their DNA-cross-linking characteristics. The tumor regrowth delay assay (Fig. 1) showed that in fact the NFSA was the less responsive of the two tumors after a single dose of 12 mg/kg *cis*-DDP. Obviously, two tumors represent a very limited sampling, and a wider range of tumor types would have to be investigated in order to determine whether the DNA damage and repair characteristics can be related to tumor responsiveness.

Despite the difficulties inherent in attempting such correlations, the possibility that tumor cells may have intrinsic differences in DNA repair capacity is nonetheless intriguing, because the excision repair pathway is known to play an important role in modulating cell sensitivity to *cis*-DDP [3, 4, 7]. Thus, even small differences in repair could have a significant impact on tumor cure, especially when the drug treatments are fractionated.

## References


1. Ando K, Hunter N, Peters LJ (1979) Immunologically nonspecific enhancement of artificial lung metastases in tumor bearing mice. Cancer Immunol Immunother 6: 151
2. Basic I, Milas L, Grdina DJ, Withers HR (1975) In vitro destruction of tumor cells by macrophages from mice treated with *Corynebacterium granulosum*. JNCI 55: 589
3. Fraval HNA, Rawlings CJ, Roberts JJ (1978) Increased sensitivity of UV-repair-deficient human cells to DNA bound platinum products which unlike thymine dimers are not recognized by an endonuclease extracted from *Micrococcus luteus*. Mutat Res 51: 121
4. Hoy CA, Thompson LH, Mooney CL, Salazar EP (1985) Defective DNA cross-link removal in Chinese hamster cell mutants hypersensitive to bifunctional alkylating agents. Cancer Res 45: 1737
5. Kohn KW, Ewig RAG, Erickson LC, Zwelling LA (1981) Measurement of strand breaks and cross-links by alkaline elution: In: Friedberg EC, Hanawalt PC (eds) DNA repair: a laboratory manual of research procedures. Dekker, New York, p 379
6. McBride WH (1986) Phenotype and functions of intratumoral macrophages. Biochim Biophys Acta 865: 27
7. Meyn RE, Jenkins SF, Thompson LH (1982) Defective removal of DNA cross-links in a repair-deficient mutant of Chinese hamster cells. Cancer Res 42: 3106
8. Milas L, Hunter N, Mason K, Withers HR (1974) Immunological resistance to pulmonary metastases in $C_3Hf/Bu$ mice bearing syngeneic fibrosarcoma of different sizes. Cancer Res 34: 61
9. Milas L, Ito H, Hunter N (1983) Effect of tumor size on S-2-(3-aminopropylamino)-ethylphosphorothioic acid and misonidazole alteration of tumor response to cyclophosphamide. Cancer Res 43: 3050
10. Milas L, Wike J, Hunter N, Volpe J, Basic I (1987) Macrophage content of murine sarcomas and carcinomas: associations with tumor growth parameters and tumor radiocurability. Cancer Res 47: 1069
11. Murray D, Jenkins WT, Meyn RE (1985) Kinetics of DNA cross-linking in normal and neoplastic mouse tissues following treatment with *cis*-platinum in vivo. Cancer Res 45: 6446
12. Murray D, vanAnkeren SC, Meyn RE (1987) Applicability of the alkaline elution procedure as modified for the measurement of DNA damage and its repair in nonradioactively-labeled cells. Anal Biochem 160: 149
13. Olinski R, Wedrychowski A, Schmidt WN, Briggs RC, Hnilica LS (1987) In vivo DNA-protein cross-linking by *cis*- and *trans*-diamminedichloroplatinum(II). Cancer Res 47: 201
14. Pinto AL, Lippard SJ (1985) Binding of the antitumor drug *cis*-dichlorodiammineplatinum(II) (cisplatin) to DNA. Biochim Biophys Acta 780: 167
15. Plooy ACM, van Dijk M, Lohman PHM (1984) Induction and repair of DNA cross-links in Chinese hamster ovary cells treated with various platinum coordination compounds in relation to platinum binding to DNA, cytotoxicity, mutagenicity, and antitumor activity. Cancer Res 44: 2043
16. Roberts JJ, Friedlos F (1987) Differential toxicity of *cis*- and *trans*-diamminedichloroplatinum (II) toward mammalian cells: lack of influence of any difference in the rates of loss of their DNA-bound adducts. Cancer Res 47: 31
17. Suit HD, Suchato C (1967) Hyperbaric oxygen and radiotherapy of a fibrosarcoma and of a squamous-cell carcinoma of C3H mice. Radiology 89: 713
18. Zwelling LA, Kohn KW (1979) Mechanism of action of *cis*-dichlorodiammineplatinum(II). Cancer Treat Rep 63: 1439
19. Zwelling LA, Anderson T, Kohn KW (1979) DNA-protein and DNA interstrand cross-linking by *cis*- and *trans*-platinum(II) diamminedichloride in L1210 mouse leukemia cells and relation to cytotoxicity. Cancer Res 39: 365


Received January 27, 1987/Accepted May 19, 1987