A new anticancer platinum compound, (−)-(R)-2-aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato) platinum(II): DNA interstrand crosslinking, repair and lethal effects in normal human, Fanconi’s anaemia and xeroderma pigmentosum cells

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Summary

Interstrand cross-linking, repair and lethal effects of (−)-(R)-2-aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato) platinum(II) (DWA2114R) were studied in normal human, Fanconi’s anaemia (FA) and xeroderma pigmentosum group A (XPA) cells. Interstrand crosslinking by DWA2114R was slower than that by cisplatin (CDDP) due to the alkylation moiety of DWA2114R. After 1 h treatment, followed by progressive interstrand crosslinking to a maximum 5 h post-incubation, CDDP induced rapidly interstrand crosslinks in ~70% DNA during the 1 h treatment, followed by a ~30% residual increase. At the maximum rate, DWA2114R was 18 times less interstrand-crosslinking than CDDP on the 1 nm basis. FA cells were specifically defective in the first half-excision of DWA2114R and CDDP-induced Pt(II) interstrand crosslinks, but XPA cells were as proficient as normal cells (t1/2 = 5–7 h). On the contrary, XPA cells were deficient in excision repair of interstrand crosslinks, but FA cells were normal. In clonogenic survival curves of all types of cells, mean lethal doses (Do) of DWA2114R were an order of magnitude greater than those of CDDP. FA cells were most (3.5 times) sensitive (Do = 25.1 ± 0.96 μM) and XPA cells were 1.9 times more sensitive (Do = 47.1 ± 1.17 μM) to DWA2114R than normal cells (Do = 87.6 ± 0.5 μM). DWA2114R and carboplatin with a cyclobutanedicarboxylato group exhibited almost similar lethal effects on each of normal, FA and XPA strains. FA (Do = 3.44 ± 0.44 μM) and XPA cells (Do = 3.84 ± 0.17 μM) were similarly 3-fold more sensitive to CDDP than normal (Do = 9.97 ± 0.15 μM). On the basis of a single lethal hit (Do), thus DWA2114R and carboplatin effectively killed more FA cells defective in interstrand crosslink repair than XPA cells defective in interstrand crosslink repair.

Cis-diaminedichloroplatinum(II) (CDDP) (Rosenberg et al., 1969) is a widely used anticancer agent which has two rapidly leaving chloro-ligands in cis- and carboplatin of a second generation derivative has a bidentate cyclobutanedicarboxylato (CBDCA) moiety replaced for dichloro-ligands (Figure 1) (Harap et al., 1980; Foster et al., 1990). Cis-Pt(II) of CDDP after rapid independent hydrolysis of chloro-ligands binds to mainly the N7 position of dG to form the first monoadduct and then bifunctional cross-linkage in a half-life (t1/2) of approximately a few hours (Eastman, 1985, 1987; Fichtinger-Schepman et al., 1985; Knox et al., 1986; Roberts & Friedlos, 1987; Lepre & Lippard, 1990). Total DNA platination by CDDP consist of 90% or more interstrandomat crosslinks in the sequences of 5'-d(dGpG)-3' (> 65%), 5'-d(ApG)-3' (20–25%) and 5'-d(ApNpG)-3' (～4%; N for any base) after in vitro reaction (Eastman et al., 1985; 1987; Lepre & Lippard, 1990). and ≤2% interstrand cross-links in DNA in vivo (Fichtinger-Schepman et al., 1985; Knox et al., 1986; Roberts & Friedlos, 1987). Interstrand crosslinking occurs between two dG residues of the complementary 5'-d(GpC)-3' sequences (Lemaire et al., 1991). A rate of in vitro DNA platination by carboplatin possessing a hydrolysisisnt resistant CBDCA group is 100 times slower than that by CDDP (Knox et al., 1986).

Recently synthesised DWA2114R is an enantiomeric isomer of (−)-(R)-2-aminomethylpyrrolidine(1,1-CBDCA)Pt(II) (Figure 1), which has the specific carrier ligand replaced for symmetric diamines in carboplatin (Morikawa et al., 1990). DWA2114R has been shown to be as potent as carboplatin in anticancer activity toward cultured tumour cells and solid tumours (Endoh et al., 1989; Morikawa et al., 1990; Matsumoto et al., 1991). Regarding side effects, CDDP causes severe neurotoxicity, myelotoxicity, and especially nephrotoxicity, which limit the therapeutic efficacy (von Hoff et al., 1979; Gildstein & Mayor, 1985). However, carboplatin currently in clinical use is devoid of nephrotoxicity, although it still causes myelosuppression (von Hoff et al., 1979; Harap et al., 1980). DWA2114R and carboplatin might be the specific carrier ligand has been shown to be less nephrotoxic and myelotoxic to administered animals than carboplatin (Endoh et al., 1989; Akamatsu et al., 1991; Matsumoto et al., 1991). Thus, DWA2114R appears to be a promising new analog.

Both Pt(II) intra- and interstrand crosslinks formed at the specific DNA sequences block DNA synthesis in vitro (Heiger-Bernays et al., 1990; Lemaire et al., 1991; Iwata et al., 1991) and in vivo (Roberts & Friedlos, 1987), and inhibit RNA transcription in vitro (Corda et al., 1991) at damaged sites. Some studies have indicated that the main cytotoxic lesions are abundant Pt(II) intranuclear crosslinks (Heiger-Bernays et al., 1990; Lepre & Lippard, 1990), while the others have indicated that the minor Pt(II) interstrand crosslinks are more lethal (Knox et al., 1986; Roberts & Friedlos, 1987). Further, G2 block through the CDDP-induced inhibition of transcription also exerts cytotoxicity (Sorensen & Eastman, 1988). Therefore, it remains to be determined which of intra- and interstrand crosslinks are more cytotoxic.

Regarding excision repair, some fraction of CDDP-induced intrastrand cross-links is removed by nucleotide excision repair, but a substantial fraction is not in mammalian cells (Ciccarelli et al., 1985; Heiger-Bernays et al., 1990; Jones et al., 1991). Especially, the major d(GpG) intranuclear crosslink is poorly repaired (Bedford et al., 1988; Page et al., 1990) and not in vitro by extracts of normal human cells (Szymkowski et al., 1992). The extract of xeroderma pigmentosum complementation group A (XPA) cells has a 5–10 fold reduced capacity to provoke repair synthesis in vitro in overall platinated plasmid DNA (Szymkowski et al., 1992). However, XPA cells were only twice as sensitive to CDDP killing, despite defective excision of CDDP-induced intrastrand crosslinks (Plooy et al., 1985), as normal cells (Plooy et al., 1985; Fujiwara et al., 1987). In this regard, we have shown that Fanconi’s anaemia (FA) cells are extraordinarily sensitive to the lethal effect of interstrand-crosslinking mitomycin C (MC) due to a selective defect in the first half.
excision of MC interstrand crosslinks compared to XPA cells (defective in only excision of MC monoadducts) and normal cells (Fujiwara et al., 1977; Fujiwara, 1982; 1983). FA cells are also more sensitive to CDDP than XPA cells (Plooy et al., 1985; Fujiwara et al., 1987). Such alternative repair defects of FA and XPA cells will enable us to distinguish the lethal effects of Pt(II) inter- and intrastrand crosslinks. Further, it is interesting to study to what extent the specific structural modifications with 2-aminoethylethylenediamine and CBDA ligands in DWA2114R affect DNA-adducting, interstrand crosslinking and lethal effect, compared to CDDP and carboplatin.

This study aimed to delineate (i) differential interstrand crosslinking between particular DWA2114R and CDDP, (ii) defective repair of Pt(II) inter- or intrastrand crosslinks in FA or XPA cells, respectively, and (iii) differential lethal effects of DWA2114R, carboplatin and CDDP on normal human, FA and XPA cells. The results show that DWA2114R with the specific structural modifications is less crosslinking and lethal than CDDP on the equimolar basis, but exerts a differentially higher lethal effect on the FA cells than on the XPA cells. Further we show, by utilising such specifically repair-defective mutants, that Pt(II) interstrand crosslinks are more lethal than intrastrand crosslinks as a basic anticancer action of DWA2114R and carboplatin.

Materials and methods

Drugs

A new derivative DWA2114R (Endoh et al., 1989) (a gift of Chugai Pharmaceutical Co., Tokyo, Japan) was studied, in comparison with the first generation CDDP (purchased from Aldrich) and second generation carboplatin (Bristol-Myers Squibb). Figure 1 illustrates their structures.

Human fibroblasts

The human diploid fibroblast strains used were: normal, NFA5 (Fujiwara, 1989) and TIG-I (courtesy of Dr T. Kaji, Tokyo Metropolitan Institute of Gerontology, Tokyo); FA, FA14TO and FA18TO (Fujiwara et al., 1977; 1987) (courtesy of Dr M.S. Sasaki, Kyoto University, Kyoto); XPA, XPHKO and XP35KO (Fujiwara, 1989). Cells were cultured in Eagle’s minimal essential medium (MEM) supplemented with 15% foetal bovine serum (FBS) (Fujiwara et al., 1977).

Detection of DWA2114R and CDDP-induced Pt(II) interstrand crosslinks

Our previous method of alkaline sucrose sedimentation (Fujiwara, 1982; 1983) was used to measure yields and repair of DWA2114R or CDDP-induced Pt(II) interstrand crosslinks because of their alkali stability (Roberts & Friedlos, 1987). The representative NFA5, FA18TO and XP35KO(A) cells were prelabelled with 111 kBq mmol⁻¹ of [5-methyl-3H]dThd (specific activity, 1.85 TBq mmole⁻¹, Amersham) for 3 days or with 7.4 kBq mmol⁻¹ of [2,4-Cd]dThd (specific activity, 1.5 GBq mmole⁻¹, New England Nuclear) for 4 days, followed by chase for 2 h prior to drug treatment. Only 3H-DNA were treated with either 0 to 2.2 mM DWA2114R or 0 to 0.333 mM CDDP in phosphate-buffered saline (PBS: 0.14 M NaCl, 3.8 mM KCl, 1.6 mM sodium phosphate, pH 7.4) at 37°C for 1 h, washed twice with PBS, and then incubated in the FBS-MEM growth medium for desired periods up to 24 h. Cells were lysed and digested with 2 mg ml⁻¹ of preheated pronase for 4 h at 37°C (Fujiwara, 1982).
Double-stranded DNA (dsDNA) in lysate was sheared optimally (see Figure 2, legend) as described previously (Fujisawa, 1983), and denatured with 0.1 N NaOH (final) for 5 min at 20°C. A 0.2-ml aliquot (~10^6 cells) was layered on the top of 4.8 ml of 5–20% (W/V) alkaline sucrose gradient, followed by centrifugation at 35,000 rpm, for 1 h at 20°C in an SW50.1 rotor of a Beckman L5-60 ultracentrifuge (Beckman Instruments) (Fujisawa, 1982; 1983). After run, each gradient was fractionated into 25 fractions, and acid-insoluble radiactivity of each fraction was counted by a liquid scintillation spectrometer. As described previously (Fujisawa et al., 1977), the weight average molecular weight (Mw) of single-stranded DNA (ssDNA) was first calculated as \( M_w = \frac{\text{M}}{\rho_{\text{M}}(370 \text{ mM})} \), where \( M \) and \( \rho \) represent molecular weight and percent radioactivity of each fraction, respectively.

When the number of Mn(II) interstrand cross-links per Da (\( = \frac{N}{Da} \)) was calculated as \( N/Da = [1/M_{n_o}] - [1/M_{n_f}] \), and the cross-link unit per average-sized molecule (N/SSDNA) was calculated as \( N/SSDNA = ([M_{n_o}]/[M_{n_f}]) - 1 \), where \( M_{n_o} \) and \( M_{n_f} \) represent Mn values of untreated control 'H-DNA (or 'C-DNA) and cross-linked 'H-DNA, respectively (Fujisawa, 1983).

### Unscheduled DNA synthesis (UDS)

Cells were grown exponentially on plastic coverslips for 2 days. UDS was measured by incubating cells for 3 h with ['H]TdTd immediately or 5 h after drug treatment. (i) For UDS during the 0 to 3 h interval immediately after treatment, cells were prelabelled for 30 min with 37 kBq ml\(^{-1}\) of ['H]TdTd (specific activity, 1.85 Td mole\(^{-1}\)) and treated with DWA2114R or CDDP (see Table I for doses used) and 2 mM HU for 1 h at 37°C, followed by labelling with 185 or 370 kBq ml\(^{-1}\) of ['H]TdTd for 3 h in the presence of 2 mM HU. (ii) For UDS during the 5 to 8 h interval of post-incubation, prelabelled cells were treated with DWA2114R or CDDP (see Table I for doses used) for 1 h, incubated with 2 mM HU for 5 h, and labelled with 185 kBq ml\(^{-1}\) of ['H]TdTd for 3 h in the presence of 2 mM HU. The cells were processed for autoradiography. Such prelabel and HU treatment eliminated miscouping of grains over cells with fortuitous residual incorporation at the very beginning and end of S phase (Fujisawa et al., 1977). Mean number of grains per cell from total counts of 30 to 50 lightly-labelled non-S cells was determined for a measure of UDS.

### Lethal effects of DWA2114R, carboplatin or CDDP

Exponentially growing normal, FA and XPA cells (see Figure 5 and Table II) were plated at appropriate densities in duplicated 60-mm plastic dishes and incubated for 4 h for attachment. For clonogenic survival, attached single cells were treated with either 0 to 300 mM DWA2114R, 0 to 250 mM carboplatin, or 0 to 35 mM CDDP in PBS for 1 h or 6 h at 37°C, washed, and incubated in FBS-MEM medium for 14 days until visible colonies of 50 cells or more developed. For the time course of the lethal effect, a fixed low concentration of either 44 mM DWA2114R (20 μg ml\(^{-1}\)) or 3.33 mM CDDP (1.0 μg ml\(^{-1}\)) was selected to treat single cells continuously for the indicated lengths of time up to 24 h in FBS-free MEM. After wash, cells were incubated for 14 days for colony assay. As all survival curves were characterised by extrapolation number \( \approx 1 \), the lethal effect was compared by only mean lethal dose (Do) of DWA2114R, carboplatin or CDDP, which resulted in the exponential region of survival curve to 1/e (37.5%).

### Results

#### Intersstrand crosslinking with DWA2114R or CDDP

[1H]TdTd-prelabelled FA18TO cells were treated with 2.2 mM DWA2114R or 0.167 mM CDDP for 1 h and further incubated for 3 and 5 h. Figure 2 shows alkaline sucrose sedimentation profiles for time-dependent interstrand crosslinking. The untreated 'H-DNA and 'H-CssDNA sedimented to form a peak of profile at fraction 15, giving an Mn(t) of 5.57 x 10^4 (Figure 2a and 2b). The 'H-DNA with the maximum number of CDDP-induced interstrand crosslinks sedimented around a peak at fraction 10, providing an Mn(t) of 1.17 x 10^4 (Figure 2b). The Mn(t) was twice the Mn(t) (= 5.57 x 10^4) of internal control 'H-CssDNA. Thus, optimal shearing of dsDNA in the mixed r.p.m. for centrifugation at 35,000 r.p.m. for 1 h avoided abnormal sedimentation of interstrand-crosslinking DNA and the condition allowed us to estimate the accurate number of interstrand crosslinks. Immediately after the 1 h treatment with 2.2 mM DWA2114R, the 'H-DNA profile shifted only slightly, while it moved progressively with post-incubation time until it approached the peak position at fraction 10 of maximally interstrand-crosslinking DNA at 5 h (Figure 2a). The bimodal profile at 5 h after DWA2114R (Figure 2a) showed that 90% DNA around the peak at fraction 10 was interstrand-crosslinked, but only ~10% around a small peak at fraction 15 remained yet uncrosslinked, providing an Mn(50) of 1.03 x 10^5 which is close to 2 x Mn(t) (= 1.14 x 10^5). The Mn(50) of Pt(II) interstrand crosslinks by DWA2114R was far less, but increased rather rapidly during a post-incubation period of 5 h. On the other hand, Figure 2b shows that 0.167 mM CDDP rapidly produced interstrand

| Table I | DWA2114R or CDDP-induced unscheduled DNA synthesis (UDS) |
| Exp* | Labelling interval | Cells (numbers counted) | UDS, mean grains ± SD cell |
|-------|-------------------|--------------------------|---------------------------|
| 1     | 0–3 h             | NFAS (n = 30)            | 9.87 ± 1.00* (100)        |
|       |                   | FA18TO (n = 30)          | 10.07 ± 1.76 (102)        |
|       |                   | XP6KO(A) (n = 30)        | 2.80 ± 1.13 (28.4)        |
| 2     | 0–3 h             | NFAS (n = 50)            | 6.50 ± 1.47 (100)         |
|       |                   | FA14TO (n = 50)          | 6.06 ± 1.48 (93.2)        |
|       |                   | FA18TO (n = 50)          | 5.68 ± 1.56 (87.4)        |
|       |                   | XP35KO(A) (n = 50)       | 0.62 ± 0.78 (9.5)         |
| 3     | 5–8 h             | NFAS (n = 50)            | 4.80 ± 1.64 (100)         |
|       |                   | FA18TO (n = 50)          | 4.72 ± 1.44 (98.3)        |
|       |                   | XP35KO(A) (n = 50)       | 0.58 ± 0.65 (12.1)        |

*Exp No. 1: 1 h-treatment with 0.22 mM DWA2114R or 0.0167 mM CDDP, followed by the initial 3 h labelling with [1H]TdTd (370 kBq/ml) in 2 mM HU and 9-day exposure. Exp No. 2: 1 h-treatment with 2.2 mM DWA2114R or 0.0167 mM CDDP, followed by the initial 3 h labelling with [1H]TdTd (185 kBq/ml) in 2 mM HU and 9-day exposure. Exp No. 3: 1 h-treatment with 0.44 mM DWA2114R or 0.05 mM CDDP, followed by a 5 h post-incubation with 2 mM HU, 3 h labelling with [1H]TdTd (185 kBq/ml) in 2 mM HU and 12-day exposure. Standard deviation of the mean. *The numerals in the parentheses indicate % UDS, relative to NFAS normal.
crosslinks in ~70% DNA immediately after the 1 h treatment, followed by ~30% additional interstrand crosslinking during the subsequent 5 h. With the doses of both agents, the 5 h post-incubation yielded the maximum of 8.5–10 interstrand crosslinks per 10⁹ Da in the FA18TO DNA in vivo, or ~1 crosslink per unit ssDNA molecule in average size of Mₙ,d = 5.57 × 10⁴ [=(~1.1 × 10⁴/5.57 × 10⁻⁸)].

Figure 3 plots the concentration-dependent Pt(II) interstrand crosslinking in the FA18TO DNA. Rates of interstrand crosslinking immediately after the 1 h treatment were 1.7 crosslinks/10⁸ Da per 1 mM DWA2114R (Figure 3a) and 4.7 crosslinks/10⁸ Da per 0.1 mM CDDP (Figure 3b). Thus, DWA2114R is ~28 times less efficient than CDDP immediately after the 1 h treatment. Rates of the maximum interstrand crosslinking at 5 h of postincubation were 5.7 crosslinks/10⁸ Da per 1 mM DWA2114R (Figure 3a), and 5.4 crosslinks/10⁸ Da per 0.05 mM CDDP (Figure 3b).

Table II Mean lethal doses (Do) of DWA2114R, carboplatin and CDDP

| Component of survival curve | Cell strain | DWA2114R | Do ± SD (µm) | Carboblatin | CDDP |
|-----------------------------|-------------|-----------|-------------|-------------|------|
| First component             |             |           |             |             |      |
| Normal                      |             |           |             |             |      |
| NFAS                        | 87.4 ± 3.98 | 79.0 ± 4.55 | 10.0 ± 0.00 |             |      |
| TIG-1                       | 88.0 ± 11.30| 86.7 ± 5.70 | 9.93 ± 0.23 |             |      |
| Mean                      | 87.6 ± 5.65 | 82.3 ± 6.18 | 9.97 ± 0.15 |             |      |
| FA                          |             |           |             |             |      |
| FA18TO                     | 25.4 ± 0.93 | 25.5 ± 0.71 | 3.90 ± 0.14 |             |      |
| FA14TO                     | 25.3 ± 2.08 | 25.0 ± 1.00 | 3.13 ± 0.15 |             |      |
| Mean                      | 25.4 ± 0.96 | 25.2 ± 0.83 | (0.30)      | 3.44 ± 0.44 | (0.35) |
| XPA                        |             |           |             |             |      |
| XP35KO                     | 48.8 ± 1.50 | 41.7 ± 2.89 | 3.93 ± 0.11 |             |      |
| XP6KO                      | 45.8 ± 2.20 | 42.0 ± 3.61 | 3.70 ± 0.14 |             |      |
| Mean                      | 47.1 ± 2.55 | 41.8 ± 2.97 | (0.51)      | 3.84 ± 0.17 | (0.39) |
| Second component           |             |           |             |             |      |
| Normal                      |             |           |             |             |      |
| NFAS                        | 101.8 ± 5.37| 96.3 ± 8.22 | 13.6 ± 1.40 |             |      |
| TIG-1                       | 102.5 ± 3.57| 97.3 ± 7.02 | 13.4 ± 0.15 |             |      |
| Mean                      | 102.0 ± 4.42| 96.7 ± 7.11 | 13.5 ± 0.90 |             |      |
| FA                          |             |           |             |             |      |
| FA18TO                     | 63.0 ± 2.08 | 75.0 ± 7.07 | 7.95 ± 0.07 |             |      |
| FA14TO                     | 66.3 ± 1.50 | 72.3 ± 3.30 | 7.83 ± 0.45 |             |      |
| Mean                      | 64.3 ± 2.73 | 73.4 ± 4.78 | (0.75)      | 7.88 ± 0.33 | (0.58) |
| XPA                        |             |           |             |             |      |
| XP35KO                     | 80.7 ± 2.08 | 84.0 ± 5.29 | 8.87 ± 0.42 |             |      |
| XP6KO                      | 80.3 ± 1.53 | 81.3 ± 8.05 | 9.50 ± 0.71 |             |      |
| Mean                      | 80.5 ± 1.64 | 81.5 ± 7.04 | (0.84)      | 9.12 ± 0.58 | (0.66) |

*Mean Do ± SD of each cell strain from three to five times repeated survival curves. *Mean Do ± SD of the two strains each of normal, FA and XPA. *The numbers in parentheses are the FA/normal and XPA/normal ratios of Do of each agent. *P = 0.005 by Student's t-test. *P = 0.003 by Student's t-test.

Repair of interstrand crosslinks

Figure 4 illustrates the time kinetics of early progressive formation of interstrand crosslinks and the subsequent repair in NFAS (normal), FA18TO and XP35KO(A) cells. As indicated above, the initial levels of interstrand crosslinks immediately after a 1 h treatment were low (~1.5/10⁸ Da) for 2.2 mM DWA2114R (Figure 4a) and high (~6/10⁸ Da) for 0.167 mM CDDP (Figure 4b) in all types of cells. Interstrand crosslinking progressed during the subsequent post-
Repair of intrastrand crosslinks by excision repair

For UDS assay, cells were treated with DWA2114R (0.22, 0.44 and 2.2 mm) or CDDP (0.0167, 0.05 and 0.167 mm) for 1 h and then radioactivity labelled for 3 h with [3H]dThd during the 0–3 h and 5–8 h intervals after treatment (see Table I, legend). The average numbers of Pt(II)-induced grains per nucleus in both intervals after DWA2114R and CDDP were small even in normal cells (Table I), as reconciled with poor repair of intrastrand crosslinks (see Introduction). The XPA (XP6KO, XP35KO) cells showed a lower level of 9.5–28% UDS of normal during both labelling intervals (Table I), indicating a defect of the XPA cells in excision repair of intrastrand crosslinks and monoadducts. However, FA18TO and FA18TO cells were normal in UDS (∼100%) in both labelling intervals (Table I). Figure 4 and Table I indicate clearly that the FA cells have a defect in the repair of Pt(II) interstrand crosslinks, but the XPA cells have a defect in excision repair of Pt(II) intrastrand crosslinks.

Differential lethal effects of DWA2114R, carboplatin and CDDP

Figures 5a to c show the biphasic clonogenic survival curves of 1 h-treated representative cells, for comparison of the lethal effects of DWA2114R, carboplatin and CDDP. Table II summarizes Do ± SD values of the two strains each of the normal, FA and XPA genotypes. All of the first components covered the major range of 80–90% kills (Figure 5). The following characteristics emerge from the first component Do values in Figure 5 and Table II. (i) The Do values of DWA2114R and carboplatin were similar and approximately an order of magnitude greater than those of CDDP in all types of cells (Figure 5 and Table II). (ii) The interstrand crosslink repair-defective FA cells and the intrastrand crosslink repair-defective XPA cells were hypersensitive to DWA2114R, carboplatin and CDDP (Figure 5), indicating that both inter- and intrastrand crosslinks are lethally acting. (iii) Toward CDDP (Figure 5c and Table II), the FA (mean Do = 3.44 ± 0.44 μM) and XPA cells (mean Do = 3.84 ± 0.17 μM) were similarly three times more sensitive than the normal (mean Do = 9.97 ± 0.15 μM). This balance of CDDP killing suggest, taking into account the alternative repair defects in the FA and XPA cells, that minor intrastrand crosslinks are more lethal than abundant intrastrand crosslinks, when not repaired. (iv) Toward DWA2114R killing
(Figure 5a and Table II), the FA cells (mean Do = 25.4 ± 0.96 μM) were most sensitive (3.5 times), and the XPA cells (mean Do = 47.1 ± 2.55 μM) were 1.9 times more sensitive than the normal cells (mean Do = 87.6 ± 5.65 μM). (v) Such situations were more or less similar for carboplatin (Figure 5b). Compared to the DWA2114R killing, however, the FA cells were slightly less sensitive to carboplatin (P = 0.003), while the XPA cells were slightly more sensitive to it (P = 0.005), including the second components (Figure 5 and Table II). Next, the second components of residual survival (<20%) for all types of cells were less steep than the first (Figure 5 and Table II). Since the number of interstrand crosslinks (presumably total Pt(II)-adducts to DNA) increases as a linear function of concentration (Figure 3), the less steep second components (Figure 5) suggest that the excess numbers of inter- and intrastrand crosslinks by ≥7 μM DWA2114R and carboplatin or ≥7 μM CDDP may be less lethally acting. Nonetheless, those lethal effects of the three agents followed a roughly similar tendency as described above for the first components.

Further, we studied the differential lethal effects of continuous treatment with a fixed low dose of 44 μM DWA2114R or 3.33 μM CDDP (approximately Do of XPA in Table II). Figure 6 shows a biphasically increasing lethal effects with time, indicating that DWA2114R and CDDP exerted 90% or more lethal effects within the initial 6 h. Again, FA18TO cells were most sensitive, and XP35KO(A) cells were immediately more sensitive to DWA2114R than normal cells (Figure 6a). Such a treatment with CDDP resulted in the balanced killing of FA and XPA cells (Figure 6b). In conclusion, FA cells were more sensitive to DWA2114R and carboplatin than XPA cells.

Discussion

First, the present results have indicated the differential interstrand crosslinking of genomic DNA of human cells by DWA2114R and CDDP. DWA2114R with CBDDCA (Figure 1) was less monoadducting to DNA, judged by a low yield of the final interstrand crosslinks (Figure 3), as carboplatin with hydrolysis-resistant CBDDCA (Figure 1) showed two orders magnitude slower rate of in vitro DNA platination that did CDDP (Knox et al., 1986). The rate of CDDP interstrand crosslinking was faster as accounted for by a 60–70% yield immediately after 1 h treatment (Figures 2 to 4), as described previously (Roberts & Friedlos, 1987). Most of the early 1 h-products with DWA2114R were monoadducts (Figure 3), while once they were formed, interstrand crosslinking progressed more rapidly (τl ≈ 2 h for pre-crosslinks) in the normal, FA and XPA cells (Figure 4). Maximum interstrand crosslinking of carboplatin in cells with both arms was observed at 5 h of the post-incubation period (Figure 4), as found with CDDP (Eastman, 1985; Knox et al., 1986; Fujinara et al., 1987). The maximum rate indicated that a molar concentration of DWA2114R 18 times that of CDDP was required for producing the equal numbers of interstrand crosslinks (Figure 3). Thus, the common hydrolysis-resistant CBDDCA group in both carboplatin and DWA2114R (Figure 1) is rate-limiting in the DNA adduction. The in vitro binding rate of the second Pt(II) arm of carboplatin has been shown to be slow with τl = 13 h (Knox et al., 1986; Roberts & Friedlos, 1987). However, the interstrand crosslinking rate in vivo of carboplatin (Knox et al., 1986) and DWA2114R (τl = 2 h in Figure 4) approaches that of CDDP in vivo. Thus, the CBDDCA leaving group becomes more labile after monoadducting to DNA.

We studied the repair of Pt(II) inter- and intrastrand crosslinks. Interstrand crosslink repair is a two-step process involving the first half excision of one arm of Pt(II) interstrand crosslinks and the subsequent removal of half-excised monoadducts (Fujinara et al., 1977; Fujinara, 1983). Unilateral arms of DWA2114R or CDDP-induced Pt(II) interstrand crosslinks were unhooked with τl = 5–7 h in normal cells (Figure 4). This τl is three times slower than that (τl = 2 h) for MC interstrand crosslinks (Fujinara, 1982). This difference could arise from different accessibility of repair enzymes to Pt(II) and MC interstrand crosslinks. The FA cells (FA18TO, FA14TO) were also almost completely defective in the first half excision of Pt(II) interstrand crosslinks, but XPA cells were proficient in that process (Figure 4) (Fujinara et al., 1987). Excision repair of Pt(II) interstrand crosslinks in mammalian cells is poor (Ciccarelli et al., 1985; Heiger-Bernays et al., 1990). Particularly, the major dGpG intrastrand crosslinks are refractory to excision repair by normal human cell extract (Szymkowski et al., 1992). Thus, a small amount of repair synthesis may arise by other intrastrand lesions. Furthermore, the amount of UDS induced by abundant intrastrand crosslinks in proficient normal and FA cells was limited (Table I), as previously indicated with MC (Fujinara et al., 1977) and CDDP (Plooy et al., 1985). XPA had a 5–10-fold reduced UDS after DWA2114R and CDDP (Table I), as observed in repair synthesis in platinated M13 DNA using the XPA cell extract (Szymkowski et al., 1992).

The lethal effect was different between DWA2114R or carboplatin and CDDP. The equitoxicity in terms of Do required a concentration of DWA2114R or carboplatin in order of magnitude greater due to reductions in DNA adducting (see above) and pharmacodynamic uptake into cells (Akamatsu et al., 1991; Knox et al., 1986), than that of CDDP (Figure 5 and Table II). Further, in phase with the maximum crosslinking at 5 h (Figure 4), more than 90% kills of FA and XPA cells by DWA2114R and CDDP were attained within 6 h (Figure 6), indicating the tight association with early production of 90% more lethally acting DNA lesions. The killing efficiencies of Pt(II) inter- and intrastrand crosslinks are also different. Do of CDDP was similar between intrastrand crosslink repair-defective XPA and interstrand crosslink repair-defective FA cells (Table II), indicating that the abundant former and the minor latter lesions remaining unrepaired exert the balanced killing, which is reflective of a higher killing efficiency of interstrand crosslink. In addition, preferential repair in actively transcribed genes is not so distinct for both Pt(II) inter- and intrastrand crosslinks (Jones et al., 1991). The completely excision-defective XPA cells were not so sensitive (at most 2-fold) to DWA2114R, carboplatin and CDDP (Figure 5) as to UV (Fujinara et al., 1977). Similarly, the XPA cells exhibited only a 1.5 to 2 times slight hypersensitivity to MC and monofunctional decaarbamoyl mitomycin C, despite the 30-
fold MS supersensitivity of the FA cells by effective blocks to replication by interstrand crosslinks (Fujiiwara et al., 1977; Kano & Fujiwara, 1981). Amounts of MC and Pt(II) interstrand crosslinks in the cell DNA are 2–10%, compared to 90% or more interstrand crosslinks. Therefore, the XPA cells can tolerate a great amount of monoadducts and interstrand crosslinks, but a replication-dependent apoptosis repair pathway, even though the major d(GpG) interstrand crosslinks effectively block replication in vitro (Ciccarielli et al., 1985; Heiger-Bernays et al., 1990; Lemaire et al., 1991; Iwata et al., 1991). In consequence, interstrand crosslinks are more potentially lethal than interstrand adducts.

In the first component of survival curves, low doses of DWA2114R killed more FA cells than XPA cells (Figures 5 and 6). This particular aspect can be discussed further taking into account that the hypersensitive lethal fractions in FA and XPA cells arise by unrepaird inter- and intrastrand crosslinks, respectively. First, the relative lethal effects in terms of the FA/normal ratios of the first component Do are identically 0.3 for DWA2114R, carboplatin and CDDP (Table II). Thus, the different Do concentrations of the three agents will produce a single hit, or the identical lethal number of interstrand crosslinks in the DNA of FA cells. However, the XPA/normal ratios are different: 0.54 for DWA2114R, 0.51 for carboplatin and 0.39 for CDDP (Table II). These figures suggest that the number of interstrand crosslinks per lethal hit in the XPA cells is less for DWA2114R and carboplatin than CDDP. Alternatively, it is suggested that, on the basis of a single lethal hit (Do), a relative ratio of highly lethally acting interstrand crosslinks over interstrand crosslinks is greater with DWA2114R and carboplatin than CDDP.

Finally, the structural modification with CBDDCA in DWA2114R and carboplatin may play a similar role in the reduction of mainly the pharmacodynamic uptake and DNA binding, but the 2-aminoethylpyrrolidine moiety in DWA2114R may not greatly affect DNA-addinguct. Instead, the 2-aminoethylpyrrolidine moiety in DWA2114R would be related to a greater improvement of nephrotoxicity and myelosuppression, compared to carboplatin without that particular carrier ligand.

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