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# Relationship between chromatin bridges in anaphase and chromosomal aberrations induced by TMP + UVA (365 nm) in CHO cells

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**Abstract**

In a recent paper, the hypothesis of 'conservative pairing' between complementary DNA strands belonging to both sister chromatids has been proposed as a phenomenon that could account for, at least in part, sister chromatid pairing in late $G_2$/mitosis. The hypothesis was verified through a cytogenetic approach, studying the so-called 'sister chromatid chromatin bridges' (SCCBs), induced in the previous $G_2$/mitosis by a crosslinking (TMP + UVA 365 nm) treatment in CHO cells (Rizzoni, M., E. Cundari, P. Perticone and B. Gustavino (1993) Chromatin bridges between sister chromatids induced in late $G_2$ mitosis in CHO cells by trimethylpsoralen + UVA, Experimental Cell Res., 209, 149–155; [1]). The purpose of the present paper is the study of the relationship between chromatin bridges without fragments in ana–telophase, which were demonstrated to be SCCBs, and chromosomal aberrations, in order to investigate their mechanism of induction. The evolution along the time of the two classes of mitotic anomalies was studied and a comparison was carried out to verify whether the bridges rise as a direct and immediate effect of the treatment or represent the misrepair-mediated effect of it. The present data show that single bridges without fragments come from a direct effect of photoinduced crosslinks in late $G_2$/mitosis. Moreover TMP + 365 nm UVA treatment shows an S-dependent clastogenic effect. The proposed hypothesis of 'conservative pairing' between DNA strands of sister chromatids is further supported.

*Keywords:* Psoralen + UVA-induced crosslink; Anaphase bridge; Chromosomal aberration

## 1. Introduction

The effect of combined treatment of psoralens + UVA is being widely investigated and several studies are available in the literature aimed at the elucidation of the mechanism for mutation induction: actually, the induced mutations appear to be highly targeted on the non-transcribed DNA strand, which appears to be repaired with a lower efficiency than the transcribed DNA strand [2–4]; it has also been found that the repair of psoralen-induced crosslinks appears to be coupled to transcription in CHO cells [4]. The suggestion that chromosome breakage occurs during the repair period after PUVA treatment has also been proposed [2].

Trimethylpsoralen is a potent photosensitizing agent which intercalates in the dark into the DNA

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minor groove and, following UVA irradiation, covalently binds to DNA, resulting in thymine photocycloaddition. UVA irradiation at 405 nm results in monoadducts only, while, when a 365-nm-wavelength UVA is used, both mono- and biadducts (interstrand crosslinks) are induced [5]. In a recent paper [1] chromatin bridges between sister chromatids (SCCB) observed in ana–telophases were induced by a trimethylpsoralen + 365 nm UVA (TMP + UVA) treatment, in order to verify a hypothesis of 'conservative pairing' occurring between DNA strands of sister chromatids. In fact, biadducts induced by such treatment covalently stabilize the possible DNA conservative pairing. The induced chromatin bridges were observed only immediately after the TMP + 365 nm UVA treatment; moreover, they were demonstrated to take place exclusively between sister chromatids, from the analysis of c-anaphases [1].

Anaphase chromatin bridges belong to the well-known phenomenon of chromosome stickiness, which was already described in the 1940s from studies on chromosomal damage induced by ionizing radiations as well as by chemical agents (for a classical review on chromosomal aberration studies see [6]). Much debate has then followed on the nature of the 'sub-chromatid exchanges' observed in c-metaphase chromosomes of cells treated in early prophase, which corresponded to the 'side-arm bridges', also called 'pseudo-chiasmata', when the chromosomal damage was observed in anaphase. The controversy was on the true nature of these exchanges for which three hypotheses were proposed: (1) that 'half chromatids' were involved in the exchange, i.e., a single strand of the double helix (the 'half-chromatid' hypothesis) [7–9]; (2) that a varying number of sub-chromatid strands were involved [10]; (3) that apparent subchromatid exchanges were masked chromatid exchanges, which actually evolved into chromosome-type aberrations at the subsequent mitosis [11,12]. Recently, the 'half-chromatid' hypothesis has been re-examined and a molecular model proposed which implies the intermediate of hairpin configuration occurring in palindromic regions of the condensing chromosome [13].

In the present work, an analytical study is carried out on the time course evolution of anaphase chromatin bridges with and without fragments, as well as a comparison with the chromosomal aberrations observed in c-metaphase at several time intervals after crosslinking induction, in a stabilized Chinese hamster cell line (CHO-K1). The purpose is to verify whether SCCBs are a direct and immediate effect of the crosslinking treatment (TMP + UVA), and distinguish them from repair-mediated and late effects, i.e., chromosomal aberrations: a late effect is expected after a treatment with an S-dependent clastogenic agent, such as psoralens + UVA treatments [14].

## 2. Materials and methods

### 2.1. Culture conditions and TMP treatment

A stabilized near-diploid cell line of Chinese hamster, CHO-K1, with a modal chromosome number of 21 was used. Cells were routinely cultivated in Ham F-12 (Gibco), supplemented with 10% fetal calf serum (Gibco), 1% L-glutamine (Flow), 2% penicillin (5000 I.U./ml) and 2% streptomycin (5000 μg/ml) (Flow).

For the experiments, cells were seeded at a low density and treated after 12 h, so as to ensure treatment of an exponentially growing cell population. Trimethylpsoralen (Trioxsalen, Sigma; CAS registry number: 3902-71-4) was added to the cell cultures at the final concentration of 1 μM; after incubation for 1 h in the dark, TMP was removed and cells were washed with medium; then, UVA irradiation (365 nm) was performed at the dose of 80 $J/m^2$, using a monochromatic lamp (Spectroline EN-14/F, Spectronix), at the dose rate of 3.77 $J/m^2 \times s$. Cells were fixed at different time intervals, starting from 1 h after irradiation up to 24 h, with a methanol/acetic acid (3:1) mixture and stained with Giemsa. Permanent slides were prepared for scoring.

### 2.2. Analysis of ana–telophases

After seeding onto coverslips, cells were fixed directly in Petri dishes without hypotonic treatment and stained with Giemsa. Besides the TMP + UVA (PU) treatment, three kinds of control treatments were performed: TMP alone (P), 365 nm UVA alone

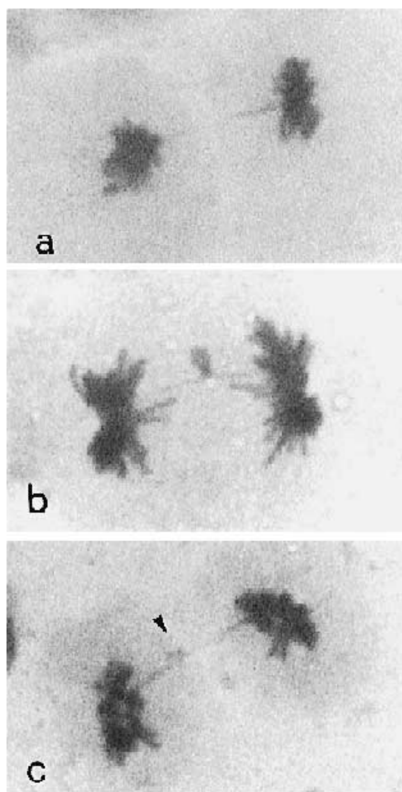


Fig. 1. Examples of chromatin bridges in ana–telophase. a: single chromatin bridge without fragment (F−). b: single F− chromatin bridge of the side-arm type, according to the classification of Dulout and Olivero [20]. c: single F+ chromatin bridge; a double fragment is shown (arrowhead). Scale bar: 10 μm.

(U) and no treatment (−). The analysis of chromatin bridges, with or without fragment, was carried out on 200 ana–telophases per point, at fixation times of 1, 2, 4, 6, 8, 13 h after the end of the treatment (Fig. 1).

*2.3. Analysis of c-metaphases*

(1) For the analysis of chromosomal aberrations, 2 h before fixation colchicine (Carlo Erba) was supplied to the cultures at the final concentration of 5 μM. Air-drying-fixed and Giemsa-stained slides were prepared from cells. TMP + UVA treated cell cultures (PU) were fixed at 2, 4, 6, 8, 15 h, 17 h 40 min, 20 h 20 min and 24 h after the end of treatment. Controls were performed with TMP (P), 365 nm UVA (U) and no treatment (−) and fixation was performed at 8, 15 and 24 h (U), 15 and 24 h (P) and 8 h (−) after the end of treatment. Chromatid-type (ct: symmetric and asymmetric exchanges, triradials) and chromosome-type (cm: dicentrics and rings) aberrations, as well as isochromatids (iso) were analysed on 200 c-metaphases per point.

(2) In order to detect whether cells had performed DNA replication during the time interval between the end of the treatment and fixation, duplicate cultures were set up in which BrUdR (Boehringer Mannheim) was added at the final concentration of 10 μM immediately after the end of the treatment. Some slides were stained following the Hoechst 33258 plus Giemsa (FPG) technique [15], so as to distinguish metaphase cells that had performed at most one round of DNA replication ($M_1$) from those that had performed two rounds ($M_2$). These slides were studied for the same chromosomal aberration analysis as described above on the following experimental points: TMP + 365 nm UVA treated cells (PU) fixed at 6, 8, 15 h, 17 h 40 min, 20 h 20 min and 24 h; controls: TMP (P), 365 nm UVA (U) and no treatment (−) fixed at 8, 15 and 24 h (P) and 15 and 24 h (U, −). Moreover, the same analysis was carried out on 160 $M_1$ and 160 $M_2$ c-metaphases fixed at 24 h. An evaluation was also carried out on the relative frequencies of $M_2$ cells at all fixation times.

(3) Finally, an immunocytochemical technique employing anti-BrUdR antibodies plus peroxidase (Cell Proliferation Kit, RPM-20, Hamersham) followed by a slight Giemsa (1%) staining, was used for the detection of mitotic cells, that were in S-phase at the time of treatment. This method was used for staining some of the slides obtained from cultures harvested at 6 (PU) and 8 h (PU, P, U), and allowed the detection of BrUdR-positive (presumptive $M_1$: MB +) mitotic cells. Thus, an estimation could be carried out of the relative frequencies of mitotic cells which had performed some DNA replication in the presence of BrUdR (MB + cells) in the time interval between the treatment and fixation.

## 3. Results

The absolute frequencies of chromatin bridges with (F +) or without (F −) fragments in ana–telophase are shown in Table 1; chromosomal aber-

Table 1
Frequencies of chromatin bridges with fragments (F+) and without fragments (F−) on 200 anaphases per point detected at different fixation times after TMP+365 nm UVA and control treatments

| Fixation time | PU | | P | | U | | − | |
|---|---|---|---|---|---|---|---|---|
| (h) | F+ | F− | F+ | F− | F+ | F− | F+ | F− |
| 1 | 2 | 22 [a] | 1 | 5 | 0 | 4 | | |
| 2 | 1 | 10 | | | | | | |
| 4 | 1 | 4 | | | | | | |
| 6 | 2 | 3 | | | | | | |
| 8 | 6 | 4 | | | | | 1 | 6 |
| 13 | 10 | 7 | 1 | 6 | 1 | 4 | 1 | 3 |

PU, TMP+365 nm UVA; P, TMP alone; U, 365 nm UVA alone; −, no treatment.
[a] Significant at 1% by $\chi^2$-test.

ration frequencies are shown in Table 2, where a distinction between chromatid- (ct) and chromosome- (cm) type aberrations as well as isochromatids (iso) is made. In addition, a detailed analysis is given on the frequencies of chromosomal aberrations in $M_1$ and $M_2$ c-metaphases at 24 h (Table 3), where the same distinction of aberration types is shown. Table 4 shows the relative frequencies of MB + mitotic cells, detected by the anti-BrUdR immunostaining technique (see Section 2: Materials and methods), at the fixation times of 6 and 8 h, together with the relative frequencies of $M_2$ mitoses observed from 6 h up to 24 h after treatment.

The time course of TMP + UVA-induced chromosomal aberrations is not significantly influenced by BrUdR administration, as shown in Table 2, which indicates that the compound does not interfere with the crosslinking treatment for chromosomal aberration induction.

A remarkable and significant ($p < 0.01$) increase from control values is observed for F− chromatin bridges (i.e., without fragments) in ana–telophase at the earliest fixation time (1 h after treatment) followed by a rapid decrease, reaching control values starting from 4 h. Conversely, the frequency of F+ chromatin bridges increases staring from 8 h after treatment (Table 1) and reach the maximum value at 13 h after treatment, where a significant difference from control values ($p < 0.01$) is reached. At the fixation time of 8 h, the frequency values of chromatid-type aberrations are rising from controls and

Table 2
Frequencies of chromosomal aberrations on 200 c-metaphases observed at different fixation times after TMP+365 nm UVA and control treatments

| Fixation time | BrUdR (−) | | | | | | | | | | | | BrUdR (+) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PU | | | P | | | U | | | − | | | PU | | | P | | | U | | | − | | |
| | ct | cm | iso | ct | cm | iso | ct | cm | iso | ct | cm | iso | ct | cm | iso | ct | cm | iso | ct | cm | iso | ct | cm | iso |
| 2 h | 1 | 2 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 4 h | 1 | 2 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 6 h | 4 | 1 | 1 | | | | | | | | | | 2 | 2 | 1 | | | | | | | | | |
| 8 h | 6 | 1 | 2 | | | | 1 | 2 | 2 | 1 | 2 | 2 | 4 | 2 | 2 | 1 | 0 | 0 | | | | | | |
| 15 h | 9 | 1 | 4 | 1 | 2 | 1 | 0 | 2 | 1 | | | | 11 | 0 | 5 | 1 | 2 | 1 | 2 | 1 | 0 | 1 | 1 | 1 |
| 17 h 40 min | 8 | 3 | 1 | | | | | | | | | | 8 | 2 | 1 | | | | | | | | | |
| 20 h 20 min | 7 | 3 | 2 | | | | | | | | | | 12 | 1 | 2 | | | | | | | | | |
| 24 h | 6 | 4 | 3 | 1 | 2 | 0 | 1 | 1 | 0 | | | | 9 | 4 | 2 | 1 | 2 | 0 | 2 | 3 | 0 | 1 | 2 | 0 |

PU, TMP+365 nm UVA; P, TMP; U, 365 nm UVA; −, no treatment.
[a] Significant at 1% by $\chi^2$-test.

Table 3
Chromosomal aberrations in $M_1$ and $M_2$ c-metaphases (160 cells examined per point) observed at 24 h after TMP + 365 nm UVA and control treatment

| c-Metaphase | PU | | | P | | | U | | | − | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ct | cm | iso | ct | cm | iso | ct | cm | iso | ct | cm | iso |
| $M_1$ | 9 [a] | 1 | 2 | 1 | 2 | 0 | 1 | 2 | 1 | 1 | 2 | 0 |
| $M_2$ | 5 | 6 | 2 | 1 | 1 | 0 | 2 | 1 | 0 | 1 | 1 | 1 |

BrUdR was supplied to the cells immediately after treatment (see text). PU, TMP + 365 nm UVA; P, TMP alone; U, 365 nm UVA alone; −, no treatment; ct, chromatid type; cms, chromosome type; iso, isochromatids.
[a] Significant at 1% by $\chi^2$-test.

reach the highest values in the time interval between 15 h and 20 h 20 min after treatment (Table 2), where significant differences ($p < 0.01$) from controls are found. Frequency values of chromosome-type aberrations start to rise at the latest times and a slight increase is detectable at 24 h after treatment (Table 2). An increase of isochromatid aberrations is also found which follows and intermediate course between chromatid-type and chromosome-type aberrations, due to their intermediate induction pathway.

At the fixation time of 24 h, in $M_1$ cells, the frequency of chromatid-type aberrations is significantly higher ($p < 0.01$) in treated cells than in controls, while chromosome-type aberrations in treated cells have frequency values similar to controls; in $M_2$ cells a moderate increase of the frequency of both chromatid and chromosome-type aberrations is detectable in treated cells vs. controls (Table 3).

Table 4
Frequencies of MB + and $M_2$ c-mitotic cells observed on 200 cells at different fixation times after either TMP + 365 nm UVA or control treatments, and subsequent cultivation in the presence of BrUdR

| Fixation time | MB + | | | $M_2$ | | | |
|---|---|---|---|---|---|---|---|
| | PU | P | U | PU | P | U | − |
| 6 h | 156 | | | 0 | | | |
| 8 h | 183 | 186 | 180 | 0 | | | |
| 15 h | | | | 0 | 0 | 0 | 1 |
| 17 h 40 min | | | | 1 | | | |
| 20 h 20 min | | | | 4 | | | |
| 24 h | | | | 41 | 135 | 95 | 119 |

MB + cells were detected following an immunostaining technique, while $M_2$ cells were distinguished following the traditional SCD method (see text). PU, TMP + 365 nm UVA; P, TMP alone; U, 365 nm UVA alone; −, no treatment.

The percent values of MB + c-metaphases (Table 4) detected at 6 and 8 h vary from about 80 to 90%, respectively. At 24 h, $M_2$ mitotic cells reach a noticeable frequency: about 20% for treated cells vs. about 50–70% for controls.

The values of MB + c-metaphases detected at 8 h were homogeneous among treated and control cultures: this shows that a remarkable cell cycle delay has not yet taken place in treated cells. On the contrary, a cell cycle delay is well evidenced at 24 h comparing the frequency value of $M_2$ mitotic cells of treated cultures, which is much lower than that of control ones (Table 4).

As far as controls are concerned, none of them shows any relevant increase from untreated cultures with respect to both chromatin bridges in ana–telophase and chromosomal aberrations in c-metaphase. This indicates that the increased values observed in the treated cultures are due to the photoadducts induced by the TMP + UVA treatment.

## 4. Discussion and conclusions

The time course evolution of chromosomal aberrations shows a clear S-dependent clastogenic effect of the TMP + UVA treatment; this is well evidenced by the increase of chromatid-type aberrations at 6–8 h (Table 2), a fixation time which corresponds to c-metaphase cells that were in S-phase of the cell cycle at the time of treatment and have performed at least the last part of it, since almost 90% of metaphase cells result positive to the anti-BrUdR antibody (MB + cells, Table 4). The peak of chromatid-type aberrations (15 h) is followed by the appearance of chromosome-type aberrations at the latest fixation times, when about 25% of metaphase cells have performed a whole S-phase plus at least part of the previous one ($M_2$ c-metaphases, Table 4).

The comparison of chromatid-type and chromosome-type aberrations between $M_1$ and $M_2$ cells observed at 24 h after treatment (Table 3) gives a further confirmation of the S-dependent mechanism of chromosomal aberration induction by the treatment. In fact, while an increase of chromatid-type

aberrations is detectable both in $M_1$ cells (which have performed only one S-phase) and in $M_2$ cells (which have performed at least a part of a second S-phase), only in $M_2$ cells an increase of chromosome-type aberrations is detectable.

An indirect, S-dependent clastogenic effect of psoralens + UVA treatments has been shown by several authors: by Ashwood-Smith et al. [16], who employed both 8-MOP and angelicin on CHA cells and studied micronucleus induction after 25–30 h from the treatment; by Natarajan et al. [14], who observed high chromosomal aberration frequencies, after angeligin, 8- and 5-MOP treatments of hamster and human cell lines at 24 h; by Hook et al. [17], who employed 8-MOP in mouse lymphoma cells, who observed an increase of chromosomal aberrations at 8 h after treatment; a peak of chromosome-type aberrations was observed in second-division cells, while chromatid deletions were found in the first division (i.e., $M_1$). Other recent data on CHO cells are also in agreement with an S-dependent clastogenic effect of the psoralen + UVA treatment [18].

Chromatin bridges with fragments (F + ) observed in ana–telophase show the same time course as that of chromosomal aberrations in c-metaphase: their increase starts at 8 h and reaches the maximum at 13 h after treatment (Table 1); this also indicates that they are the consequence of an S-dependent clastogenic effect.

The observed F − chromatin bridges correspond to the immediate and slight stickiness proposed by Gaulden [19]. However, an immediate stickiness after treatment with S-dependent clastogens has never been described before; in fact, a late stickiness was evidenced following treatments with mitomycin C [20,21], adriamycin [20] and MNNG [22]. Their results can be explained as the consequence of a repair-mediated effect. On the other hand, chromatin bridges without fragments (F − ) represent the direct consequence of the TMP + UVA-induced crosslinks, since they already appear at their maximum observed frequency at the first fixation time (1 h) and rapidly reach control values at 4 h after treatment (Table 1). This result also supports the hypothesis that they are the consequence of crosslinks induced by the treatment in chromosomal regions where, at the time of the treatment, conservative pairing has taken place [1]. The comparison of 365 and 405 nm UVA exposure of PUVA treatments of CHO cells [1] showed that only crosslinks exhibit the direct and immediate effect of chromatin bridges between sister chromatids.

The molecular model recently proposed by Bender [13] for the understanding of the nature of subchromatid exchanges (the 'half-chromatid exchange' theory), is also in good agreement with the 'conservative pairing' hypothesis of Rizzoni et al. [1]: hairpin configurations occurring in palindromic sequences of corresponding regions of both sister chromatids may be annealed and interactions between part of the sequence give rise to a DNA pairing in a conservative fashion, in the absence of any DNA breakage event. This phenomenon is also likely to take place during the process of chromosome condensation, i.e., late $G_2$–early prophase, in the regions where repetitive sequences, such as telomeric repeats, are present.

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