Influence of irofulven, a transcription-coupled repair-specific antitumor agent, on RNA polymerase activity, stability and dynamics in living mammalian cells

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Summary

Transcription-coupled repair (TCR) plays a key role in the repair of DNA lesions induced by bulky adducts and is initiated when the elongating RNA polymerase II (Pol II) stalls at DNA lesions. This is accompanied by alterations in Pol II activity and stability. We have previously shown that the monofunctional adducts formed by irofulven (6-hydroxymethylacylfulvene) are exclusively recognized by TCR, without involvement of global genome repair (GGR), making irofulven a unique tool to characterize TCR-associated processes in vivo. Here, we characterize the influence of irofulven on Pol II activity, stability and mobility in living mammalian cells. Our results demonstrate that irofulven induces specific inhibition of nucleoplasmic RNA synthesis, an important decrease of Pol II mobility, coupled to the accumulation of initiating polymerase and a time-dependent loss of the engaged enzyme, associated with its polyubiquitylation. Both proteasome-mediated degradation of the stalled polymerase and new protein synthesis are necessary to allow Pol II recycling into preinitiating complexes. Together, our findings provide novel insights into the subsequent fate of the stalled RNA polymerase II and demonstrate the essential role of the recycling process for transcriptional reinitiation and viability of mammalian cells.

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Key words: Transcription-coupled repair, RNA Polymerase II, DNA lesions, Recycling, Living mammalian cells

Introduction

Nucleotide excision repair (NER) is a remarkably flexible DNA repair pathway capable of eliminating a wide range of DNA lesions caused by environmental genotoxins and cancer chemotherapeutic agents (Reardon and Sancar, 2005). Mutations in one of the many genes involved in the NER process have severe consequences, as illustrated by three well-known genetic disorders, xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (Lehmann, 2003). NER is divided into two subpathways: global genome repair (GGR), responsible for repair of transcriptionally inactive regions of the genome as well as the nontranscribed strands of expressed genes, and transcription-coupled repair (TCR), involved in the repair of the transcribed strand of active genes (Hanawalt, 2002).

TCR initiates when the elongating RNA polymerase stalls at DNA lesions (Lainé and Egly, 2006a). In mammalian cells, only the presence of stalled RNA polymerase II (Pol II) has been associated with the activation of TCR, whereas the genes transcribed by RNA polymerases I and III (Pol I and Pol III) are unlikely to be repaired through this pathway (Christians and Hanawalt, 1993; Dammann and Pfeifer, 1997). Besides Pol II, TCR requires at least five additional proteins, the Cockayne syndrome A and B factors (CSA and CSB) and the XP-associated proteins XPB, XPD and XPG (Lainé and Egly, 2006b). CSA is a protein that has a strong potential to interact with different protein complexes and might serve as a molecular chaperone (Lainé and Egly, 2006b; Fousteri et al., 2006), whereas CSB is believed to bind to the arrested Pol II and to recruit TFIIH, an essential ten-subunit protein complex that includes the DNA helicases XPB and XPD (Coin and Egly, 1998; Tantin, 1998; Fousteri et al., 2006). CSA and CSB are specific for TCR. By contrast, XPB, XPD and XPG play a role in both TCR and the overall NER pathway. In all types of NER, XPB and XPD are required to create a 'bubble' of ~30 nucleotides in the DNA surrounding the lesion (Evans et al., 1997). The bubble then serves as a substrate for the XPG and ERCC1/XPF endonucleases (Mu et al., 1996; Evans et al., 1997). Rare mutations in the genes encoding XPB, XPD or XPG can result in Cockayne syndrome in addition to XP (Lehmann, 2003). The precise function of the XP factors in TCR is only partly understood. However, recent data suggest that XPG, through its noncatalytic domains, plays an important role in the recognition of the stalled Pol II and the activation of CSB, whereas TFIIH seems to be able to change the conformation of the bound polymerase, thus rending the DNA lesion more available to the repair machinery (Sarker et al., 2005).

A particularly interesting question is what happens to the stalled Pol II. Numerous studies have reported that the arrested RNA

polymerase II large subunit (Pol II LS) becomes polyubiquitylated and degraded following exposure to cisplatin or UV irradiation (Bregman et al., 1996; Ratner et al., 1998; Jung and Lippard, 2006), and it was generally assumed that this degradation was essential for the repair process (Svejstrup, 2002; Yang et al., 2003). More recent data indicate that degradation of the Pol II LS is not an absolute requisite for repair of cisplatin adducts, at least in model systems using cellular extracts from human cells (Tremeau-Bravard et al., 2004; Sarker et al., 2005; Lainé and Egly, 2006a). Furthermore, yeast mutants lacking ubiquitin ligase, and thus unable to ubiquitylate the stalled Pol II LS, are still TCR proficient (Lommel et al., 2000). Furthermore, it has been shown that proteolysis of the Pol II LS can occur independently of the TCR process as treatment with alpha-amanitin or nucleotide starvation is accompanied by ubiquitylation and proteolysis of the polymerase (Lee et al., 2002; Yang et al., 2003). These findings suggest that it might be the stalling of the elongating Pol II that triggers its ubiquitylation and proteolysis rather than the TCR process as such. Interestingly, the 26S proteasome is physically associated with gene regions that correspond to the locations of Pol II build-up in yeast (Gillette et al., 2004). The proteasome has also been shown to localize at active transcription sites in human cells (Iborra et al., 2004), suggesting an important role for this protein complex in the transcriptional process.

In TCR-deficient fibroblasts, cells undergo apoptosis in response to UV irradiation or cisplatin treatment (Ljungman and Zhang, 1996; Dumaz et al., 1997; Ljungman et al., 1999). It was originally assumed that the induction of apoptosis was linked to the accumulation of stalled polymerases as CS-deficient cell lines were unable to polyubiquitylate and degrade Pol II LS (Bregman et al., 1996; McKay et al., 2001). However, others have shown that only the kinetics of Pol II LS degradation differ between CS-deficient and -proficient cell lines, making the correlation between Pol II degradation, DNA repair and induction of apoptotic cell death less straightforward (Luo et al., 2001).

A major limitation for studying TCR-associated processes in living cells is the lack of TCR-specific agents. We (Koeppel et al., 2004) and others (Kelner et al., 1994; Jaspers et al., 2002) have reported that irofulven (6-hydroxymethylacylfulvene), a monofunctional covalent DNA binder (Woynarowski et al., 1997), and structurally related compounds induce DNA adducts that are exclusively recognized by TCR, but not by GGR. Here, we report that exposure of mammalian cells to irofulven is associated with potent and specific inhibition of nucleoplasmic RNA synthesis, an important decrease of Pol II mobility and a time-dependent loss of Pol II LS linked to its polyubiquitylation and proteasome-mediated degradation. Furthermore, we demonstrate that degradation of Pol II LS is necessary for transcriptional recovery following irofulven treatment by removing the engaged and stalled polymerase, thus permitting recycling. Accordingly, administration of proteasome inhibitors was associated with increased sensitivity to irofulven, suggesting that the accumulation of immobilized polymerases combined with the inhibition of transcriptional recovery contribute to the induction of irofulven-mediated cell death.

Results

Irofulven exposure is associated with potent and specific inhibition of nucleoplasmic RNA synthesis

The incorporation of radiolabeled uridine reveals that RNA synthesis is reduced by 50% after 1 hour of exposure of HeLa cells to 1 μg/ml irofulven (Fig. 1A). Interestingly, irofulven concentrations as high as 5 μg/ml never inhibited the incorporation by more than 75%, suggesting that irofulven selectively inhibits certain types of polymerases (Fig. 1A).

To investigate this further, the influence of irofulven on nascent RNA synthesis was determined by bromo-UTP incorporation. Actinomycin D is a specific inhibitor of nucleolar transcription at low concentrations (0.2 μg/ml) and becomes a general inhibitor at higher concentrations (Fig. 1B). By contrast, DRB, a Pol II inhibitor, has no effect on nucleolar incorporation but reduces the nucleoplasmic signal (Fig. 1B). Irofulven behaves like DRB, identifying irofulven as a specific inhibitor of nucleoplasmic transcription (Fig. 1B). Additional studies carried out with bromouridine, that gives access to both mitochondrial and nucleoplasmic RNA synthesis, showed that irofulven only inhibits nucleoplasmic incorporation and had no influence on mitochondrial RNA synthesis, even at concentrations as high as 5 μg/ml (Fig. 1C).

Irofulven exposure is accompanied by decreased Pol II LS dynamics in mammalian cells

Pol II dynamics was monitored using fluorescence loss in photobleaching (FLIP) on a cell line stably expressing functional Pol II LS tagged with the green fluorescent protein (GFP-Pol II) (Sugaya et al., 2000; Kimura et al., 2002). Results obtained with untreated cells are consistent with the existence of two main populations of Pol II, a major one that diffused rapidly, and a minor one that exchanged slowly (Fig. 2A). The first population corresponds to the noncommitted fraction of Pol II LS, whereas the second represents the fraction of engaged enzyme (Kimura et al., 2002). DRB increases the fast fraction, presumably by releasing the transcriptionally active polymerase from the DNA template (Fig. 2A). Conversely, actinomycin D, a DNA intercalator, used at a concentration of 5 μg/ml, is capable of generally inhibiting transcription (Fig. 1B), stalls the polymerase and decreases the fast fraction (Fig. 2A). Irofulven also increases the slow, engaged fraction of Pol II (Fig. 2A) in a dose-dependent manner, with a maximal effect at 10 μg/ml (supplementary material Fig. S1). These results suggest that irofulven treatment leads to stalling of elongating polymerases without interfering with transcriptional initiation, thus increasing the overall fraction of engaged enzyme.

As irofulven and DRB had opposite effects, the influence of the two drugs together was determined. The results were comparable to those obtained with DRB alone (Fig. 2A), indicating that the influence of irofulven is not due to any covalent binding between the enzyme and DNA but, rather, a result of increased levels of elongating and/or initiating Pol II.

Irofulven exposure is associated with a time-dependent loss of Pol II LS

Previous results showed that the appearance of stalled Pol II LS after UV or cisplatin treatments was followed by Pol II LS degradation (Bregman et al., 1996; Ratner et al., 1998; Jung and Lippard, 2006). To determine whether irofulven is able to induce Pol II LS degradation, HeLa cells were exposed to the drug for 15 to 180 minutes. Whole-cell extracts were prepared and subjected to immunoblotting with a monoclonal antibody that binds specifically to the N-terminus of Pol II LS (Besse et al., 1995) (Fig. 2B). The immunoreactive band is characteristically broad because this antibody recognizes hyperphosphorylated (II_O), hypophosphorylated (II_A) and intermediate forms of Pol II LS (Fig. 2B, lane 0). Interestingly, after only 15 minutes of irofulven exposure, the fraction of the II_O form of Pol II LS increased from

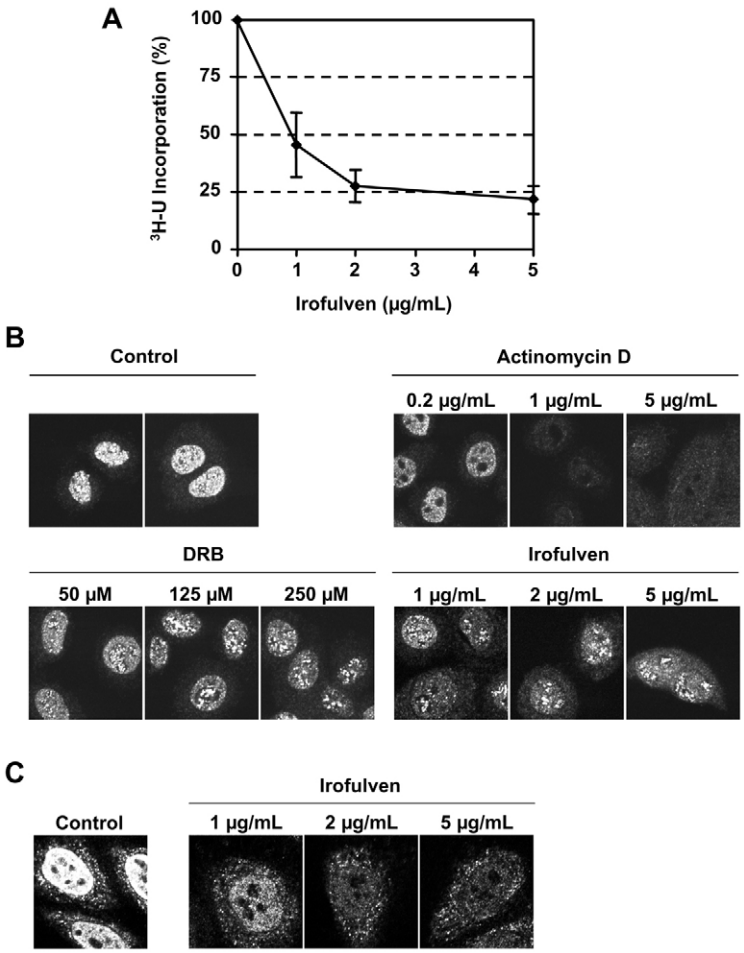

Fig. 1. Influence of irofulven on RNA synthesis. (A) HeLa cells were incubated with the indicated concentrations of irofulven for 1 hour and the influence on RNA synthesis was measured by incorporation of radiolabeled uridine. Error bars represent standard errors. (B) HeLa cells were exposed for 1 hour to actinomycin D, DRB or irofulven at the indicated concentrations, permeabilized and the engaged polymerases allowed to extend their transcripts in the presence of bromo-UTP. Nascent RNAs were revealed by immunolabeling of bromo-labeled nucleotides. (C) HeLa cells were exposed for 1 hour to irofulven at the indicated concentrations. 15 minutes before fixation, bromo-uridine was added to the medium. Bromo-labeled RNAs were revealed by immunolabeling.

38 to 52%, to reach >60% by 1 hour (Fig. 2B, compare lanes 0, 15 and 60). The increase in the II_O form is accompanied by the depletion of the II_A form, which almost completely disappeared after 180 minutes of irofulven exposure. This is consistent with our FLIP data, which indicated that, despite stalling of the polymerase at the lesion (Fig. 2A), initiation can continue, resulting in an overall increase in the engaged fraction (II_O) and thereby loss of the II_A form of the enzyme.

Pol II LS contains a C-terminal region with 52 heptapeptide repeats. During initiation, the Ser5 residue of this repeat is phosphorylated, whereas Ser2 becomes phosphorylated later, during elongation (Svejstrup, 2004). The monoclonal H14 antibody specifically recognizes phosphorylated Ser5, whereas the H5 antibody specifically recognizes phosphorylated Ser2 (Bregman et al., 1995). To further define the II_O form of Pol II LS in irofulven-treated cells, cellular extracts were subjected to immunoblotting with both antibodies. The results show that the H5-immunoreactive band is lost rapidly following irofulven treatment, whereas the H14 band increases (Fig. 2B, second and third panels). This suggests that the relative loss of II_A is mainly due to an accumulation of initiating, rather than elongating, polymerases.

For cells exposed to irofulven for longer than 1 hour, the total amount of Pol II LS decreased (Fig. 2B). This loss was preceded by the appearance of a smear above the II_O form, which was visible after only 15 minutes and became maximal by 30 minutes (Fig. 2C). Further blotting with the H5 and H14 antibodies showed the appearance of discrete bands above the II_O form in irofulven-treated cells (supplementary material Fig. S2). These results suggest that irofulven induces post-translational modifications of Pol II LS in living cells, followed by Pol II LS degradation.

Irofulven induces polyubiquitylation and proteasome-mediated proteolysis of Pol II LS

Further experiments were conducted to establish a possible involvement of the ubiquitin pathway. The results show that affinity-purification leads to increased amounts of the H14-reactive species in treated but not in control cells, indicating that irofulven induces polyubiquitylation of Pol II LS (Fig. 3A). Similar results were obtained with the 7C2 and H5 antibodies, although to a lesser degree (data not shown).

For further confirmation, Pol II LS from irofulven-treated and control cells was immunoprecipitated and the resulting blots probed with an antibody against ubiquitin. Again, a specific labeling was apparent in treated but not in control cells (Fig. 3B). Together, these results strongly suggest that Pol II LS is ubiquitylated in response to irofulven exposure.

Next, the role of the proteasome was examined. Addition of MG-132, a widely used proteasome inhibitor, completely abolished the irofulven-associated degradation of Pol II LS (Fig. 3C). However, MG-132 is equally known as a potent inhibitor of thiol proteases (Adams et al., 1998). Therefore, the influence of velcade (bortezomib/PS-341), a potent and specific inhibitor of the chymotrypsin-like activity of the proteasome (Adams et al., 1998), was also determined (Fig. 3D). Velcade efficiently blocked the irofulven-induced degradation of Pol II LS for at least 6 hours at concentrations as low as 20 nM. Together, these findings indicate that the proteasome pathway plays a major role in the degradation of Pol II LS in living cells following irofulven exposure.

Interestingly, the engaged form of Pol II LS is preferentially degraded in irofulven-treated cells (Fig. 3E). DRB treatment, which induces the disappearance of the hyperphosphorylated form of Pol II LS in favor of II_A (Dubois et al., 1994) and leads to the release of the transcriptionally active polymerase from the DNA template (Fig. 2A), clearly decreases the irofulven-associated loss of Pol II LS (Fig. 3E). These findings indicate that degradation of the Pol II LS is, at least partially, dependent on its transcriptional status.

Velcade blocks transcriptional reinitiation and recycling of Pol II LS

To determine whether proteasome-mediated degradation of Pol II LS plays a role in transcriptional reinitiation, we assessed the influence of proteasome inhibitors on the transcriptional recovery following irofulven treatment (Fig. 4A). As previously shown (Fig. 1), 1 hour of irofulven exposure (1 μg/ml) significantly inhibits transcription (Fig. 4A, time 0). Subsequent post-incubation in drug-

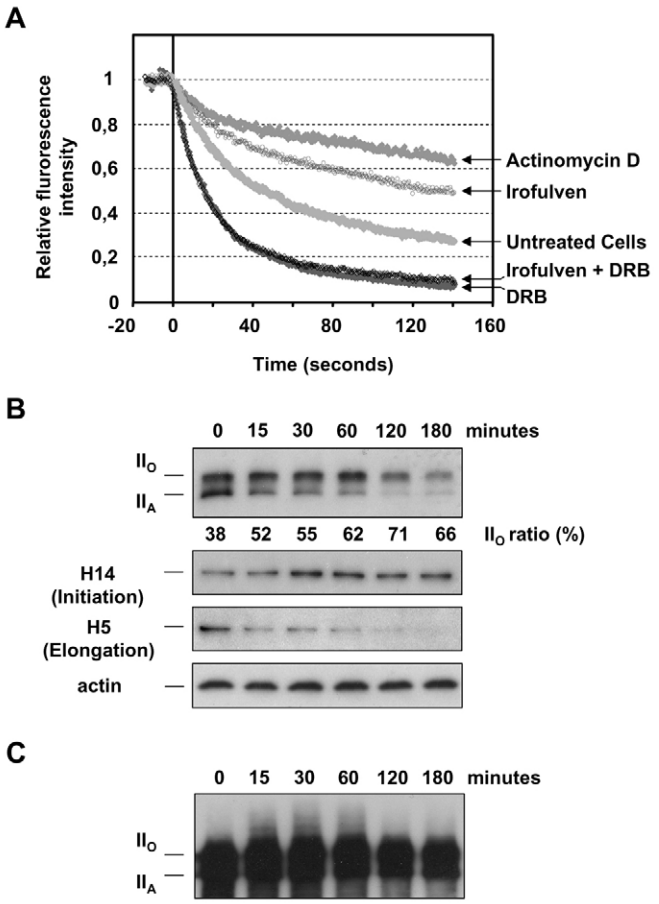

Fig. 2. Influence of irofulven on the dynamics of Pol II LS and induction of post-translational modifications. (A) Mammalian cells expressing GFP-Pol II were subjected to FLIP analysis under different experimental conditions: untreated cells, cells treated with 100 μM DRB, cells treated with 5 μg/ml actinomycin D, cells treated with 5 μg/ml irofulven and cells treated with 5 μg/ml irofulven and 100 μM DRB together. All curves represent an average of at least 12 independent measurements. (B) HeLa cells were incubated with 1 μg/ml irofulven for the indicated times. Total (upper panel), H14-reactive (second panel) and H5-reactive (third panel) levels of Pol II LS were assessed by immunoblotting, with actin (last panel) serving as an internal control for equal loading. Band intensities were measured, and the indicated II_O ratios were calculated as [(II_O/total Pol II LS)×100]. (C) Overexposed Pol II LS immunoblot.

free media was accompanied by a gradual recovery, and, by 6 hours, the transcriptional activity was fully restored. However, the recovery was totally blocked if the irofulven-treated cells were post-incubated in the presence of velcade, this happening even though velcade on its own only marginally influenced the transcriptional activity (Fig. 4A). This suggests that, in the presence of stalled polymerase, either DNA repair is not functional or Pol II LS is not recycled. The first hypothesis is difficult to assess in vivo, although in vitro data have demonstrated that degradation of Pol II LS is not an absolute requisite for successful repair, at least with regard to cisplatin-induced DNA lesions (Tremeau-Bravard et al., 2004; Sarker et al., 2005; Jung and Lippard, 2006). By contrast, the second hypothesis can be investigated by determining the respective levels of H14 and H5 labeling (Fig. 4B and supplementary material Fig. S3).

Velcade on its own did not influence the relative levels of H14 and H5 immunoreactive bands (supplementary material Fig. S3), in agreement with its modest effect on bromouridine incorporation (Fig. 4A), whereas irofulven exposure induced important modifications of both H5 and H14 levels (Fig. 4B and supplementary material Fig. S3). Interestingly, the H5 curve resembled the transcriptional reinitiation curve (compare Fig. 4B, right panel, and Fig. 4A). After 1 hour of treatment with irofulven, H5-immunoreactive material decreased (Fig. 4B, right panel, closed symbols, time 0), in agreement with our previous observations (Fig. 2B). After 6 hours of post-incubation in drug-free media, the initial levels were restored.

The H14 profile appeared more complex. First, irofulven induced a marked increase (Fig. 4B, left panel, closed symbols, time 0), indicating that transcriptional initiation was unaffected (Fig. 2B). After irofulven removal, a subsequent decrease of H14 labeling was observed (Fig. 4C, left panel, closed symbols, time 1 and 2) most probably due to either polymerase degradation or to restored capacity to initiate elongation. After 4 hours post-incubation, an increase of H14 labeling is observed (Fig. 4C, left panel, closed symbols, time 4 and 6), which precedes the subsequent recovery of transcriptional activity (Fig. 4A).

Importantly, all these changes were absent if the irofulven-treated cells were post-incubated in velcade (Fig. 4B, open symbols). H5 labeling was not significantly altered, whereas H14 labeling increased markedly until a plateau was reached, suggesting that the initiating Pol II cannot proceed to elongation in the presence of velcade. The capacity of velcade to sustain a constant level of engagement of Pol II after irofulven treatment was further confirmed by FLIP (data not shown).

Recovery of basal levels of Pol II LS following irofulven treatment is dependent on novel protein synthesis

The results above indicate that irofulven exposure induces an important loss of the II_A form of Pol II LS, most likely due to the accumulation and/or degradation of Pol II LS phosphorylated counterparts. The presence of II_A is an absolute requisite to allow transcriptional reinitiation (Svejstrup, 2004). Short exposure times, however, do not induce a complete loss (Fig. 4C, left panel). Upon irofulven removal, the total amounts of Pol II increase and the initial equilibrium between the II_A and II_O forms is gradually restored. In clear contrast, when irofulven-treated cells are post-incubated in the presence of cycloheximide (Fig. 4C, right panel), an inhibitor of protein synthesis, Pol II LS is rapidly and completely lost within 2 hours. This is clearly due to the combined effects of irofulven and cycloheximide rather than to cycloheximide as such as cycloheximide alone required ~14 hours to reduce the cellular levels of Pol II LS by 50% (data not shown). These findings indicate that new protein synthesis plays an important role in the recycling of Pol II LS following irofulven treatment and is required for transcriptional recovery.

Cytotoxic activity of irofulven depends on the proportion of engaged RNA polymerase

Previous findings suggest a close correlation between the absence of transcriptional recovery and the induction of apoptotic cell death after exposure to UV irradiation or cisplatin (McKay et al., 2001). To assess whether this is also valid in our experimental model, we determined how velcade influences the cellular toxicity of irofulven. The results (Fig. 4D) show that 6 hours post-incubation of irofulven-treated cells with velcade increases irofulven cytotoxicity. By contrast, post-incubation with DRB decreases cytotoxicity, suggesting that it is not inhibition of RNA synthesis as such that influences the cytotoxicity but, rather, the prolonged presence of

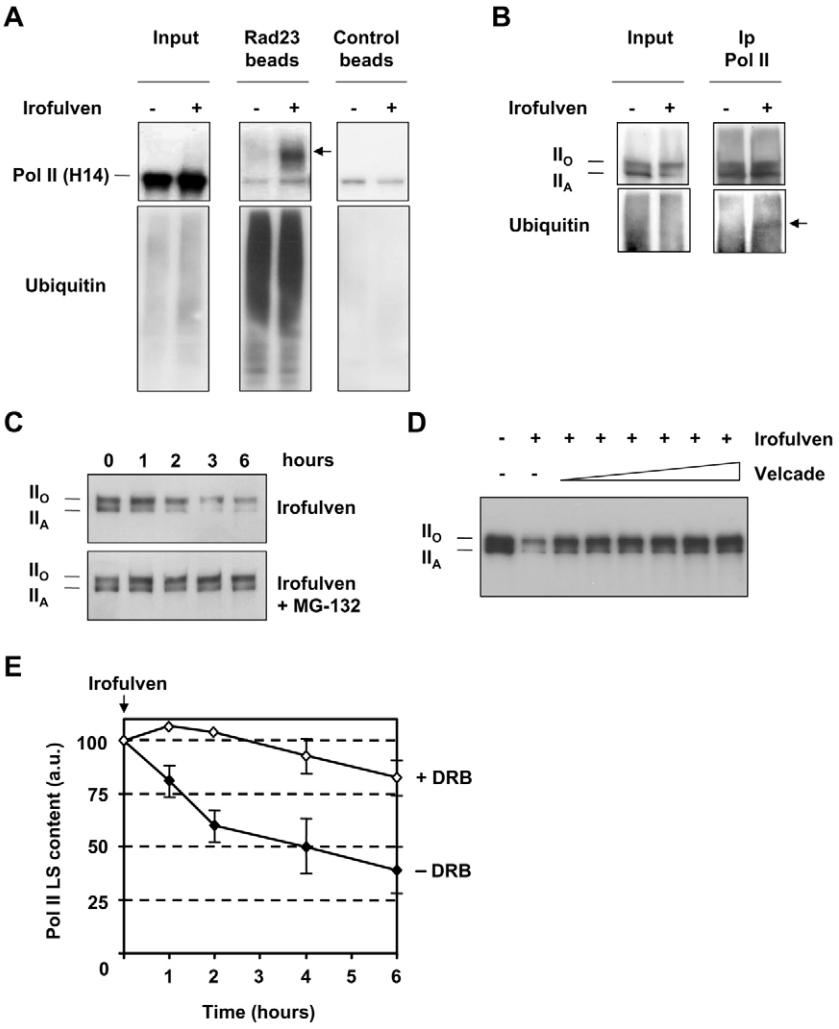

Fig. 3. Irofulven treatment is accompanied by polyubiquitylation of Pol II LS followed by its proteasome-mediated proteolysis. (A) HeLa cells were incubated in the absence or presence of 1 μg/ml irofulven for 30 minutes, polyubiquitylated proteins were affinity purified and the resulting proteins were subjected to gel electrophoresis and the blots probed with the H14 monoclonal antibody. (B) HeLa cells were incubated in the absence or presence of 1 μg/ml irofulven for 30 minutes, Pol II LS was immunoprecipitated and the resulting proteins were subjected to gel electrophoresis and the blots probed with the P4D1 monoclonal antibody against ubiquitin. (C) HeLa cells were treated for the indicated times with 1 μg/ml irofulven in the absence (upper panel) or presence (lower panel) of 5 μM MG-132. (D) HeLa cells were treated for 6 hours with 1 μg/ml irofulven in the absence or presence of increasing amounts of velcade (bortezomib/PS-341) ranging from 20 to 1000 nM. (E) HeLa cells were incubated with 1 μg/ml irofulven for the indicated times in the presence (◇) or absence (◆) of 100 μM DRB. The total amounts of Pol II LS were assessed by immunoblotting and the relative intensities measured and standardized with respect to actin levels. Error bars represent standard errors and are indicated when they exceed symbol size.

engaged Pol II LS. Velcade, by inhibiting the degradation of Pol II LS, reinforces the cytotoxic effect, whereas DRB, by facilitating Pol II release, diminishes it. The relatively modest influence of DRB on the overall cytotoxicity is most likely because its effects on Pol II are counterbalanced by the accumulation of unrepaired DNA lesions due to lack of functional TCR (Sarker et al., 2005; Lainé and Egly, 2006a).

Discussion

Irofulven is an antitumor covalent DNA binder derived from the fungal product illudin S. The illudins are the only compounds identified so far that are exclusively repaired by TCR without involvement of GGR (Kelner et al., 1994; Jaspers et al., 2002; Koeppel et al., 2004), making them unique tools to probe TCR-associated processes in living cells.

Here, we report that irofulven specifically inhibits nucleoplasmic RNA synthesis and induces strong inhibition of Pol II dynamics in living cells. The absence of GGR as well as the lack of effect on Pol I strongly suggest that the DNA-irofulven adducts do not perturb the local DNA architecture in the same way as other bulky adducts. Interestingly, both the hypophosphorylated (II_A) and hyperphosphorylated (II_O) forms of Pol II LS are affected by exposure to irofulven. Immediately following irofulven treatment, accumulation of the engaged II_O form is accompanied by a rapid loss of the II_A form, as shown by both FLIP and immunoblotting, indicating that the stalling of the elongating polymerase at the DNA-irofulven adducts does not prevent further initiation (Fig. 5). Irofulven exposure is accompanied by polyubiquitylation and proteasome-mediated proteolysis of Pol II, similar to what has been reported for UV and cisplatin (Bregman et al., 1996; Ratner et al., 1998; Jung and Lippard, 2006).

Use of phosphorylation-specific monoclonal antibodies recognizing either the initiating (H14) or elongating (H5) forms of Pol II LS allowed us to further characterize the irofulven-induced changes of the II_O fraction. The results show that the H5 fraction rapidly disappeared, whereas the H14 fraction accumulated, suggesting that the enrichment of the II_O fraction is mainly due to an accumulation of initiating, rather than elongating, polymerases (Fig. 5, lower-right panel). One way to explain the quick loss of the H5 form could be that it is a better substrate for polyubiquitylation than the H14 form. In support of this hypothesis, it has been shown that Ser5 phosphorylation in yeast (recognized by the H14 antibody) inhibits polyubiquitylation, whereas Ser2 phosphorylation (recognized by the H5 antibody) does not (Somesh et al., 2005). However, in mammalian cells, numerous data, in addition to ours, show that the H14 species are also polyubiquitylated (Ratner et al., 1998; Inukai et al., 2004; Jung and Lippard, 2006), indicating that the preferential disappearance of the H5 form can not only be due to differences in levels of polyubiquitylation. A second way to explain the quick loss of the elongating form compared with the initiating form could be that the probability to hit an irofulven adduct is greater at elongation than at initiation, thus, leading to the recruitment of the repair machinery, which is followed by rapid degradation of the large subunit of the polymerase. Interestingly, in contrast to the elongating form of Pol II, the initiating form is only lost after prolonged irofulven exposure, which means that it is eventually degraded. The degradation of the H14 species might occur because it hits a lesion in the vicinity of the promoter region, or, alternatively, because the initiating polymerase is unable to leave the promoter region. The initiating polymerase is then

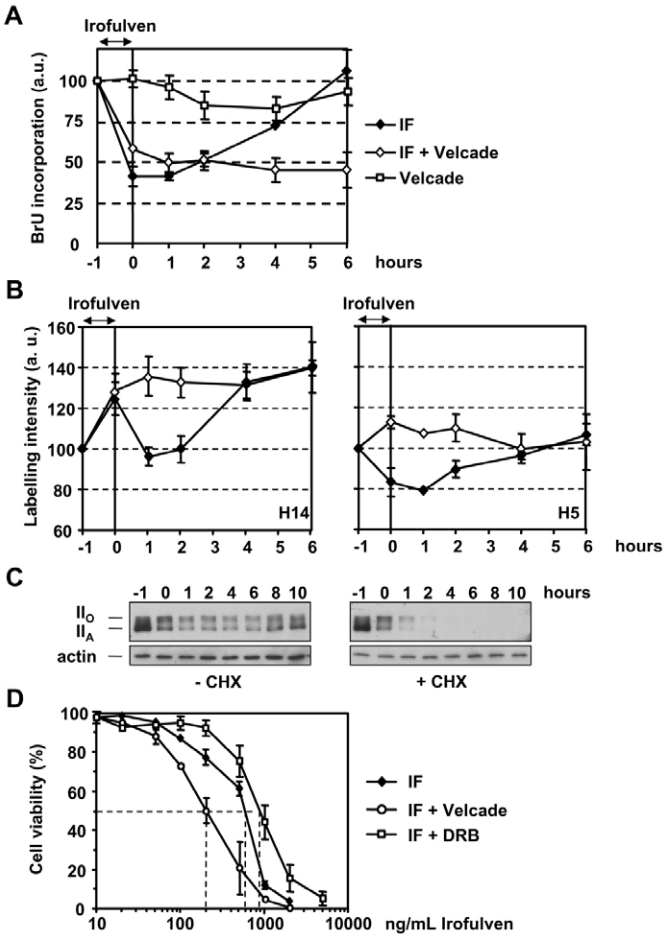

Fig. 4. Degradation of Pol II LS is required for transcriptional restart following irofulven-exposure and influences cell viability. (A) HeLa cells were incubated for 30 minutes in the presence (◇, □) or absence (◆) of 150 nM velcade before addition of 1 μg/ml irofulven (time –1). After 1 hour of irofulven exposure, cells were post-incubated for the indicated times in the presence (◇) or absence (◆) of 150 nM velcade. Cells continuously exposed to velcade alone were included as control (□). Bromo-uridine was added for the last 15 minutes of incubation and the cells were fixed. Nascent RNA was revealed by immunolabeling, and the intensities were measured. All curves represent the average of at least three independent experiments in which the intensities of bromo-uridine labeling were assessed for more than 100 cells per time-point. Error bars represent standard errors and are indicated when they exceed symbol size. (B) The same conditions as above. The relative contents of H14 (left panel) and H5 (right panel) species were assessed by immunoblot and the relative intensities of each band measured and standardized versus actin. All curves represent an average of three independent experiments. Error bars represent standard errors and are indicated when they exceed symbol size. Filled symbols: cells treated for 1 hour with 1 μg/ml irofulven and post-incubated in drug-free medium; open symbols: cells pre-, co- and post-incubated with 150 nM velcade. (C) HeLa cells were preincubated for 30 minutes in the absence (left panel) or presence (right panel) of 10 μg/ml cycloheximide (CHX) followed by coincubation with 1 μg/ml irofulven for 1 hour. Cells were then post-incubated for the indicated times in the absence or presence of 10 μg/ml cycloheximide, and the relative content of Pol II LS forms was assessed by immunoblotting. Actin was included as a control for equal loading (lower panels). (D) HeLa cells were treated with irofulven for 1 hour, incubated in drug-free media for 5 days, and cellular viability was assessed by MTT assay. ●, cells treated with irofulven and post-incubated in drug-free medium; ○, cells pre-, co- and post-incubated with 500 nM velcade; □, cells pre- co- and post-incubated with 100 μM DRB. All curves represent an average of at least three independent experiments, each done in duplicate. Error bars represent standard errors and are indicated when they exceed symbol size.

polyubiquitylated and degraded in response to the stalling, this being similar to what has been reported for alpha-amanitin (Lee et al., 2002).

The disappearance of elongating polymerases, despite accumulation of H14-reactive species, strongly suggests that initiating Pol II cannot proceed efficiently to elongation (Fig. 5, lower-right panel). One possibility could be that, following the loss of the elongating Pol II LS, the other subunits forming the Pol II holoenzyme, as well as elongating factors, are not physically recycled towards the promoters where new H14 species accumulate, thus interfering with transcriptional resumption by inhibiting elongation. A second attractive possibility could be that factors involved in both transcription and repair become preferentially associated with repair sites. It is well established that TFIIH plays a dual role in transcription and repair through its two helicase activities, XPB and XPD. In particular, it has been shown that TFIIH plays a crucial role in promoter clearance after transcriptional initiation (Kumar et al., 1998; Seroz et al., 2000; Pal et al., 2005). In the absence of TFIIH, transcription can still initiate in vitro, but the transcription complex encounters a block to elongation (Kumar et al., 1998). This is reminiscent of in vivo studies demonstrating that, following transcriptional initiation, Pol II pauses in a region proximal to the promoter (Bentley and Groudine, 1986; Akhtar et al., 1996). To explain this general phenomenon, it has been suggested that the concentration of TFIIH in vivo is limiting (Kumar et al., 1998). We therefore propose that DNA-irofulven adducts sequester TFIIH through the TCR pathway, thus preventing promoter clearance. This idea is further enforced by results obtained with living UV-irradiated cells, where the equilibrium between bound and unbound TFIIH rapidly shifts towards bound TFIIH involved in DNA repair (Hoogstraten et al., 2002). Interestingly, more and more proteins (e.g. CSB) seem to have dual functions in repair and transcription (Fousteri et al., 2006; Proietti-De-Santis et al., 2006). We therefore propose that the direct involvement of common factors during the initial step of both repair and transcription plays an important role in the decreased ability of the initiating Pol II to proceed to elongation following irofulven exposure.

Prolonged Pol II stalling might have more serious consequences for transcriptional reinitiation than for DNA repair. At least the initial steps of DNA repair can take place in the presence of a stalled polymerase (Tremeau-Bravard et al., 2004; Sarker et al., 2005; Fousteri et al., 2006; Lainé and Egly, 2006a), whereas the presence of hypophosphorylated polymerases is an absolute requisite for preinitiation (Rockx et al., 2000; Svejstrup, 2004). In agreement, the absence of the II_A form of Pol II LS due to the accumulation of II_O forms has been shown to represent a major factor hindering transcriptional recovery, even on undamaged substrates (Rockx et al., 2000). Hence, the fate of the stalled polymerases is of considerable interest. To recycle Pol II LS, at least two processes could be involved: dephosphorylation and/or degradation/resynthesis. It has been shown that dephosphorylation by the FCP1 phosphatase probably participates in recycling during normal transcription (Cho et al., 1999). However, there is currently no evidence for a direct role of the CTD phosphatases in polymerase recycling during TCR. By contrast, numerous reports show that, in mammalian cells, Pol II LS is polyubiquitylated and degraded by the proteasome following UV or cisplatin treatments (Bregman et al., 1996; Ratner et al., 1998; Jung and Lippard, 2006). Here, we show that irofulven induces clear accumulation of hyperphosphorylated Pol II LS, which is followed by its

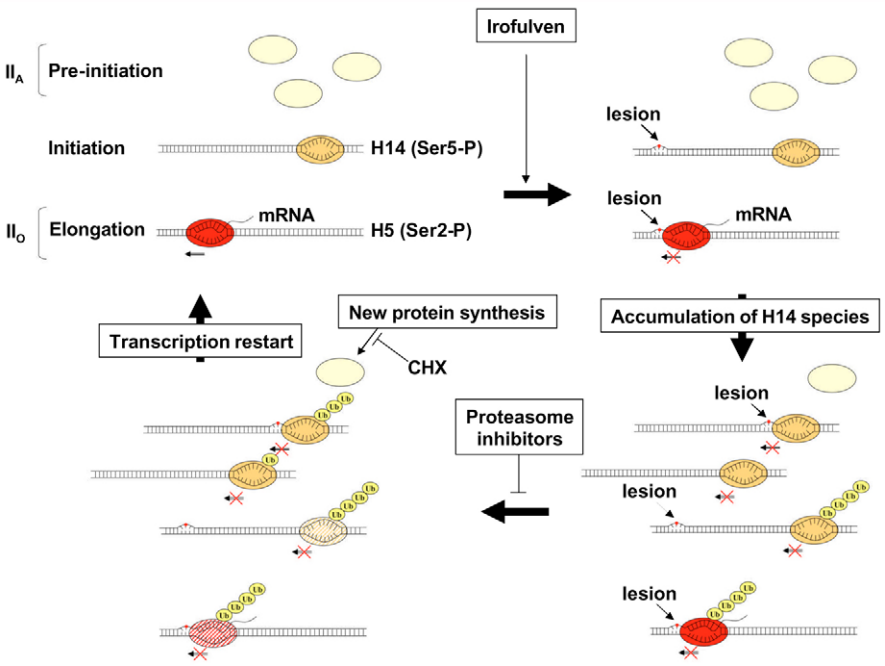

Fig. 5. Model depicting how transcription copes with irofulven lesions. Under normal conditions (upper-left panel), two main populations of Pol II coexist: the major one corresponds to a noncommitted fraction of Pol II, the second represents the fraction of engaged enzyme. Among the engaged fraction, two subtypes can be distinguished: the initiating Pol II, recognized by the H14 antibody, and the elongating subtype, recognized by the H5 antibody. The monoclonal H14 antibody specifically recognizes phosphorylated Ser5, whereas the H5 antibody specifically recognizes phosphorylated Ser2. Both serine residues are present within a heptapeptide that is repeated 52 times in the C-terminal domain of the human Pol II LS. In the presence of irofulven (upper-right panel), lesions are formed. These lesions are specifically processed by TCR after stalling of the RNA polymerase. Irofulven treatment is followed by an accumulation of initiating Pol II LS and a concomitant loss of the non- committed fraction of Pol II (lower-right panel). In contrast to the H14 species, the H5 fraction is rapidly lost. This suggests that the initiating Pol II cannot proceed efficiently to elongation. Three main hypothesis can be raised to explain this phenotype: (1) the presence of numerous lesions in the vicinity of the promoter region, (2) the inefficient recycling of other subunits that form, with Pol II LS, the holoenzyme and/or (3) the preferential association with repair sites of factors involved in both transcription and repair. Stalled polymerases become ubiquitylated and degraded through the proteasome. New protein synthesis is required to restore the initial pool of noncommitted enzyme necessary for transcription restart (lower-left panel). Proteasome inhibitors block Pol II LS recycling, inhibiting transcriptional recovery and increasing the probability of secondary DNA lesions. Cycloheximide (CHX), by blocking new protein synthesis, induces a rapid and complete loss of Pol II LS owing to the continuous degradation of newly engaged enzymes.

polyubiquitylation and degradation (Fig. 5, lower panels). Pol II degradation is specifically inhibited by two potent proteasome inhibitors, MG-132 and velcade (Adams et al., 1998). Even after prolonged exposure to these inhibitors, much of the polymerase remains hyperphosphorylated, suggesting that the enzyme is not dephosphorylated and recycled into the II_A form. In agreement, both proteasome inhibitors completely inhibited transcriptional reinitiation. New protein synthesis also plays a crucial role in the recovery of II_A levels as it can be prevented by cycloheximide (Fig. 5, lower-left panel).

Exposure to UV irradiation or cisplatin is accompanied by rapid apoptotic cell death in TCR-deficient fibroblasts (Ljungman and Zhang, 1996; Dumaz et al., 1997; Ljungman et al., 1999). However, the precise mechanism triggering cell death remains unclear (Ljungman and Lane, 2004). One possibility is that apoptosis is triggered by the prolonged transcriptional inhibition (McKay et al., 2001). However, our results make this unlikely. Indeed, coincubation with the transcriptional inhibitor DRB decreases, rather than increases, the cytotoxic activity of irofulven, whereas coincubation with velcade increases it. These findings suggest that it is the prolonged presence of stalled polymerases that triggers cell death, possibly due to collisions with the replication fork resulting in more-lethal secondary DNA lesions (McKay et al., 2002). Thus, by blocking Pol II LS recycling, we both increase the probability of secondary DNA lesions and inhibit transcriptional recovery (Fig. 5, lower-left panel). In agreement, it is becoming increasingly clear that UV irradiation does not prevent transcriptional recovery of TCR-deficient cells only because the genes are not repaired but also because there is a defect in transcriptional reinitiation (Rockx et al., 2000; Proietti-De-Santis et al., 2006). Accordingly, preliminary biochemical data, obtained in our laboratory with CSB-deficient cell lines, show that the major difference between CSB-proficient and CSB-deficient cell lines is the inability of the CSB-deficient cells to restore the initial levels of II_A after irofulven treatment. However, the precise interpretation of these findings remains complicated and requires a careful analysis. Indeed, although the CSB phenotype was believed originally to represent mainly a failure in the coupling between the elongating Pol II and NER, additional functions have now been revealed, involving a direct role for CSB at multiple levels of transcription. This includes transcriptional initiation (Rockx et al., 2000; Proietti-De-Santis et al., 2006), transcriptional elongation (Selby and Sancar, 1997; Tantin et al., 1997; Lee et al., 2001) and even transcriptional modulation of proteins linked to chromatin structure (Newman et al., 2006). Thus, further work is required to understand the exact relationship linking transcription reinitiation and repair.

In conclusion, we have used irofulven, an antitumor alkylator that is recognized by TCR, but not by GGR, to dissect TCR-associated processes in living mammalian cells. Our findings demonstrate that the fate of the stalled RNA polymerase plays a crucial role in transcriptional recovery and cellular survival. An improved understanding of TCR might provide the molecular basis to establish new pharmaceutical treatments that directly link the transcriptional machinery to induction of tumor cell death.

Materials and Methods

Chemicals and cells

Irofulven was supplied by MGI Pharma (Bloomington, MN). Velcade (bortezomib/PS-341) was sourced from Janssen-Cilag (Issy-les-Moulineaux, France), and MG-132 and DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) were purchased from Calbiochem (San Diego, CA). Protease inhibitors were obtained from Roche Diagnostics (Mannheim, Germany), and PBS and cycloheximide were sourced from Sigma (Saint-Quentin Fallavier, France).

HeLa cells were grown in Dulbecco's modified Eagle's Medium (InVitrogen, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (Perbio Science, Brebières, France). The C23 clone is derived from tsTM4 cells and stably expresses green fluorescent protein (GFP)-tagged-RNA polymerase II LS (Sugaya et al., 2000). C23

cells were cultured at 39°C in Ham's F-12 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. All growth media contained 60 μg/ml penicillin G and 100 μg/ml streptomycin (InVitrogen).

Inhibition of RNA synthesis

HeLa cells (4×10^5 cells per ml) were exposed to irofulven for 50 minutes at 37°C. Next, 5 μCi/ml of [5-^{3}H]uridine (Amersham) were added and the cells were incubated for an additional 15 minutes. Cells were washed in PBS and resuspended in 100 μl PBS. 100 μl of an ice-cold 10% trichloroacetic acid (TCA) solution were added, followed by 30 minutes incubation on ice and loading on GF/C filters (Whatman, Brentford, UK). Filters were washed with 10% TCA. To quench TCA, filters were washed in ethanol. The radioactivity was determined by scintillation counting (Liquid Scintillation Analyser Tri-CARB 2100 TR, Packard).

'Run-on' transcription with bromo-UTP

HeLa cells were plated onto coverslips and grown overnight. Transcription was monitored by allowing engaged polymerases to extend their transcripts in the presence of bromo-UTP in permeabilized cells (Pombo et al., 1999). Briefly, cells were preincubated with or without drugs for 1 hour. Cells were then washed in 'physiological' buffer at 4°C (PB: 100 mM potassium acetate, 30 mM KCl, 10 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM Na_2ATP, 1 mM dithiothreitol and RNase inhibitor, pH 7.4), permeabilized for 5 minutes at 4°C in PB containing 150 μg/ml saponin (Sigma), washed twice in PB (4°C) and incubated for 12 minutes at 33°C in transcription buffer (PB supplemented with 100 μM ATP, CTP, GTP and 5 μM bromo-UTP). Reactions were terminated by fixation, and immunolabeling was carried out as described below.

Bromo-uridine incorporation

HeLa cells were plated onto coverslips and grown overnight. For irofulven alone, cells were incubated for 1 hour at 37°C in the presence of the indicated concentrations of irofulven, 5 mM bromo-uridine (Aldrich) was added and cells were incubated for an additional 15 minutes. Alternatively, cells were preincubated for 30 minutes at 37°C in the absence or presence of 150 nM velcade. Next, irofulven (1 μg/ml final concentration) was added, followed by incubation for 1 hour. The drug-containing medium was removed and the cells were post-incubated for the indicated times in the absence or presence of 150 nM velcade. Bromo-uridine was added and cells incubated for an additional 15 minutes. After treatment, cells were immediately fixed and immunolabeled as described below.

Immunofluorescence and microscopy

Cells were fixed in 4% paraformaldehyde for 20 minutes and permeabilized with PBS-Triton (0.5%) for 20 minutes. Coverslips were saturated in 'PBS^{+}' (PBS containing 1% bovine serum albumin and 0.2% gelatin, Sigma) and antigens revealed by using a primary mouse antibody directed against IdU/BrdU (1:100 dilution; Caltag, Burlingame, CA). The secondary antibody was Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, Bar Harbor, ME). Antibodies were diluted in PBS^{+}. Images were collected using either a Radiance 2000 MP confocal microscope (BioRad Laboratories, Hemel Hempstead, UK) or a Retiga 1300 camera (QImaging, Burnaby, Canada) attached to a Nikon TS 100 microscope. Fluorescence intensities were measured using 'MetaMorph' software (Universal Imaging Corporation, Downingtown, PA). The background over noncellular regions was subtracted.

Fluorescence loss in photobleaching (FLIP)

FLIP experiments using a cell line expressing GFP-Pol II were carried out as described previously (Kimura et al., 2002). Briefly, cells stably expressing GFP-Pol II encode a temperature-sensitive mutated endogenous Pol II LS. At 39°C, their genetic defect is complemented by the expression of the tagged polymerase, demonstrating that the GFP-Pol II is fully functional (Sugaya et al., 2000). Cells were cultured at 39°C in glass-bottomed dishes (MatTek Cultureware, Ashland, MA). To determine the influence of drugs, cells were preincubated with the agent of interest for 30 minutes at 39°C before analysis. Images were collected immediately, with cells grown on the microscope stage at 39°C. Fluorescence images were collected using a confocal microscope (Radiance2000 [Bio-Rad Laboratories]: 25 mW Ar laser at 4% power, 10 zoom, pinhole aperture 4, scan speed 600 lines/second, Kalman filtration 3, LP500 filter) fitted to a microscope (model TE300; Nikon) with a 60× PlanApo objective (NA 1.4). A field with two cells was selected, imaged approximately every 0.43 seconds for 13 seconds, and the bottom half of a nucleus was bleached (100% laser power) during subsequent scans. Curves were analyzed as described previously (Kimura et al., 2002).

Affinity purification of polyubiquitylated proteins

HeLa cells (5×10^5 cells per ml) were incubated for 30 minutes at 37°C in the absence or presence of 1 μg/ml irofulven. Cells were washed twice in PBS and resuspended in lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X100, 50 mM HEPES pH 7.4, 10 mM *N*-ethylmaleimide and protease inhibitors). Cell extracts were sonicated and centrifuged at 20,800 ***g*** for 10 minutes. Protein concentrations were adjusted to 1 mg/ml. Polyubiquitylated proteins were enriched using affinity beads comprising a GST-fusion protein containing the ubiquitin-associated sequence of Rad23 conjugated to glutathione-agarose (Calbiochem, San Diego, CA). Beads (control or Rad23-conjugated) and cell extracts were coincubated for 4 hours at 4°C, washed four times and resuspended in SDS-polyacrylamide gel loading buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol and 100 mM dithiothreitol). Proteins were resolved and blotted as described below.

RNA polymerase II immunoprecipitation

HeLa cells (5×10^5 cells per ml) were incubated for 30 minutes at 37°C in the absence or presence of 1 μg/ml irofulven. Cells were washed twice in PBS and resuspended in lysis buffer (750 mM NaCl, 2 mM EDTA, 0.75% NP-40, 50 mM Tris pH 7.8, 10 mM *N*-ethylmaleimide and protease inhibitors). Cell extracts were sonicated and centrifuged at 20,800 ***g*** for 10 minutes. 1 ml of the supernatants was incubated for 4 hours while rotating on a wheel at 4°C with preconjugated dynabeads (300 μl of protein G dynabeads, Dynal-InVitrogen, Cergy-Pontoise, France, that had been conjugated overnight with 20 μg of anti-Pol II LS antibody, clone N20, Santa Cruz Biotechnology). Beads were washed four times in lysis buffer and resuspended in SDS-polyacrylamide gel loading buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol and 100 mM dithiothreitol). Proteins were resolved and blotted as described below.

Immunoblotting

HeLa cells (10^5 cells per ml) were incubated with 1 μg/ml irofulven at 37°C for the indicated times. Cells were then washed twice in PBS, counted and lysed for 30 minutes at 4°C in SDS-polyacrylamide gel loading buffer. When the influence of DRB, velcade or MG-132 on Pol II LS degradation was evaluated, cells were exposed to the drugs for 30 minutes followed by coexposure with irofulven. When the influence of velcade or cycloheximide on Pol II LS recycling was evaluated, cells were preincubated in the presence or absence of 150 nM velcade or 10 μg/ml cycloheximide for 30 minutes followed by coincubation with 1 μg/ml irofulven for 1 hour. This was followed by post-incubation in the presence or absence of velcade or cycloheximide. Proteins were resolved on a linear-gradient SDS-polyacrylamide gel (5-15%) and blotted onto PVDF membranes (Biorad). Membranes were saturated by TBST-milk (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% Tween 20 and 5% dehydrated skimmed milk) and antigens revealed by immunolabeling using the following primary antibodies: (i) mouse monoclonal IgG anti-polymerase (clone 7C2) (Besse et al., 1995), (ii) mouse monoclonal IgM anti-H5 (Covance Research Products, Berkeley, CA), (iii) mouse monoclonal IgM anti-H14 (Covance Research Products), (iv) mouse monoclonal IgG_1 anti-ubiquitin (clone P4D1, Santa Cruz Biotechnology) and (v) mouse monoclonal IgG anti-actin (clone AC-15, Sigma). The horseradish peroxidase (HRP)-conjugated secondary antibodies used were: (i) donkey anti-mouse IgG and (ii) donkey anti-mouse IgM (μ chain specific) (Jackson ImmunoResearch). Antigens were detected using an enhanced chemiluminescence kit (Amersham Biosciences). Digital images were collected, and band intensities measured using MetaMorph software.

Growth inhibition and viability assays

The cytotoxic effects of irofulven were determined by the MTT (methylthiazolyldiphenyl-tetrazolium bromide) viability assay, as described previously (Poindessous et al., 2003). For drug combinations, HeLa cells were first incubated for 30 minutes in the absence or presence of 500 nM velcade or 100 μM DRB, followed by coincubation with irofulven for 1 hour. Cells were then post-incubated for 6 hours in the absence or presence of velcade or DRB, followed by additional incubation in drug-free media for 5 days. The concentrations of velcade and DRB used were associated with approximately 20% growth inhibition when given alone.

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