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A theoretical model for p53 dynamics

Identifying optimal therapeutic strategy for its activation and stabilization

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The activation and stabilization of tumor suppressor p53 are very important in preventing cells from becoming cancerous. Hence, many experimental works have been carried out to investigate p53's dynamics through its interactions with other proteins and its therapeutic applications for the treatment of cancers. In this work, by analyzing a theoretical model, we attempt to search for an optimal therapeutic strategy that guarantees the activation and stabilization of p53. For this purpose, we introduce a new mathematical model including oncogene activation and ARF, which are recognized as crucial for tumor suppression but have not yet been considered in most theoretical works. Through mathematical modeling and numerical simulations, we confirm several important properties of p53 dynamics: the role of the oncogene-mediated activation of ARF as an important factor for the activation and stabilization of p53, the necessity of time delays in negative feedback loops to guarantee sustained p53 oscillations, and the digital behavior of p53 pulses. Furthermore, we propose that the binding of ARF to Mdm2 and enhancing the degradation of Mdm2 is an efficient strategy for therapeutic targeting, which may assure the activation and stabilization of p53.

Introduction

The tumor suppressor protein p53, often called the "guardian of the genome,"[1] is a transcription factor that regulates the expression of a large number of target genes.[2] It is normally maintained in the inactivated state at low levels in unstressed cells. However, when the cells are under stress conditions (e.g., when cells suffer DNA damage by ionizing radiation), both the level of p53 and its transcriptional activity are increased to induce the transcription of various genes in its tetrameric form. p53 thus plays a major role in diverse cellular processes such as the regulation of cell cycle, apoptosis, DNA repair, and senescence. As p53 plays a critical role in preventing cells from becoming cancerous, extensive studies have been made to control the activity and stability of p53 for therapeutic purposes, such as in the treatment of cancers.[3-6]

The level and transcriptional activity of p53 are controlled by the negative regulator Mdm2 (murine double minute 2). The E3 ligase protein Mdm2 directly binds to p53 and inhibits its transcriptional activity, favoring its nuclear export and causing its ubiquitinization and proteasomal degradation. p53 itself also stimulates an increase in Mdm2 levels. Therefore, the p53-Mdm2 feedback loop is composed of two parts: a slow (or delayed) positive part by p53-induced transcription and translation of Mdm2, and a fast negative part by Mdm2-induced protein interaction to p53. As the time delay between the increase in p53 and the increase in Mdm2 transcription in the positive part is sufficiently long, about 30~40 min, robust and sustained oscillatory behaviors of p53 and Mdm2 take place. This has been experimentally checked. Thus, the p53-Mdm2 negative feedback loop inducing such oscillatory behaviors has been considered a crucial part in the activation and stabilization of p53.

Furthermore, p53 is affected by many other upstream mediators that induce post-translational modifications of p53. For example, DNA damage activates the protein kinases ATM (ataxia-telangiectasia mutated) and Chk2. DNA damage induces rapid autophosphorylation of ATM resulting in the activation of ATM kinase. As active ATM phosphorylates both p53 and Mdm2, it activates and stabilizes p53. ATM also phosphorylates the checkpoint kinase Chk2, which directly phosphorylates p53, giving further contribution to its activation and stabilization.

There have been various attempts to understand the mechanisms of the p53-Mdm2 feedback loop and the main interactions between p53 and other proteins.[7] Many theoretical models have especially focused on the p53's oscillation under DNA damage generated by ionizing radiation.[8-18] The main results of some these theoretical works can be summarized as follows:

(1) The sustained oscillatory behaviors of p53 and Mdm2 can be induced only when the time delay in the p53-Mdm2 feedback loop is considered.[8-10] Nearly all of theoretical models consider this time delay.

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(2) The oscillation of p53 must be "digital like."[11,12] According to the work of Lahav et al.[19] neither the size nor shape of the p53 pulses in single cells; rather, the number of pulses depends on the level of the input damage signal. To include this digital behavior, Ma et al.[11] assumed high constant levels of ATM to explain the sustained oscillations.

(3) To understand better the exact behaviors of p53, any theoretical model should include protein interactions between p53 and other proteins such as ATM.[11,12] The addition of positive feedback[13-15] or negative feedback[15,16] on the p53-Mdm2 feedback loop has been considered. Batchelor et al.[16] confirmed both theoretically and experimentally that the p53-Mdm2 loop does not drive sustained p53 oscillations by itself without the aid of ATM and Chk2. They further introduced a second negative feedback loop between p53 and Wip1, containing time delay similar to the p53-Mdm2 feedback loop.

(4) Mdm2 directly binds to p53, inactivating the transcriptional activity of p53. Whereas most models assume that the degradation of p53 depends on total Mdm2 levels irrespective of whether Mdm2 is bound to p53 or not, some models[17,18] consider the p53·Mdm2 complex as an additional independent variable.

In this context, there is a need to have a new theoretical model considering all these properties. Thus, in this study, we introduce a new model considering all of the following components: the time delay in the p53-Mdm2 negative feedback loop; the digital behavior of p53; several protein interactions occurred by ATM, Chk2, and others; the second negative feedback loop between p53 and Wip1; and the p53·Mdm2 complex. In addition, we consider oncogene activation as an important stimulus to activate p53 and stress on the functional importance of the alternate reading frame (ARF) (product of the p16/INK4A locus; $p14^{ARF}$/$p19^{ARF}$) in the activation and stabilization of p53.

p53 is widely known to be activated by several stresses such as ionizing radiation, activated oncogenes, hypoxia, and chemotherapeutic drugs. According to recent experiments on mice, the response of p53 to DNA damage has little impact on cancer protection, but ARF-dependent activation of p53 is crucial for p53-mediated tumor suppression.[20-22] Nonetheless, the oncogene activation or ARF has not been strongly addressed in most theoretical works. Proctor and Gray[18] considered ARF as a protein directly dependent on DNA damage in their ARF model. However, according to widespread views, ARF is not induced in response to DNA damage.[23-25] Instead, it is believed that ARF loss can attenuate the DNA damage response through its effects on Mdm2 and p53, and conversely, ATM and its dampening effects on p53 activation can blunt the ARF response.[26,27] Thus, in our model, we do not consider ARF as induced by DNA damage.

We introduce an expanded theoretical model for p53 dynamics that includes the oncogene-mediated activation of ARF. This model is expected to describe qualitatively the oscillation of p53 in a more realistic manner. Furthermore, as p53 is a feasible target for cancer therapy, our model may be helpful in the search for an optimal therapeutic strategy that would enhance the stabilization and activation of p53.

The strategies for the therapeutic targeting of the p53 pathway can be arranged as follows:

(1) Promoting the production of wild-type p53

We can increase the amount of p53 by gene therapy,[28,29] that is, the exogenous supplementation of p53. Furthermore, by using small molecules such as PRIMA-1,[30] and MIRA-1,[31] mutant p53 can also be reactivated, restoring the p53 function in mutant *TP53*-carrying tumor cells.[32]

(2) Decreasing the levels of Mdm2

Antisense inhibition of Mdm2 by oligonucleotide in cells with wild-type p53 significantly enhances p53 activity.[33,34]

(3) Releasing p53 from Mdm2 using the inhibitors of the p53-Mdm2 interaction Small-molecule inhibitors such as Nutlins[35,36] target wild-type p53 or Mdm2, preventing p53-Mdm2 interaction and therefore inhibiting Mdm2-mediated p53 degradation.[3,37,38]

(4) Enhancement of Mdm2 degradation by ARF

ARF promotes Mdm2 degradation and indirectly increases p53 activity.[39-43]

Using our theoretical model, we investigate the most optimal therapeutic strategy for the stabilization and activation of p53. For this purpose, we make a new deterministic model with self-consistent ordinary differential equations. Although a stochastic approach is more realistic than a deterministic one,[15] results from the stochastic models show significant variations in p53 oscillations. Thus, they may not be helpful in our attempt to determine the optimal strategy for the therapeutic targeting of the p53 pathway.

Model

As previously mentioned, our model starts from the activation of oncogenes such as Myc and Ras. We follow the presumption that ARF does not mediate the activation of p53 by DNA damage. The activation of p53 can then be divided into two pathways: that induced by ionizing radiation and is independent of ARF, and that induced by oncogene activation and is dependent on ARF.[23-25] We focus on 17 interactions occurring between two stimuli (oncogene activation and ionizing radiation), DNA damage, and 6 important proteins (p53, Mdm2, ATM, Chk2, Wip1 and ARF), as shown in **Figure 1**. The 17 interactions are enumerated as follows:

(1) The activation of oncogene Myc[44,45] or Ras[46,47] induces ARF expression.

(2) Oncogene activation triggers DNA damage in pre-cancerous lesions.[48-52]

(3) Ionizing radiation also causes DNA damage.[2]

(4) Induced DNA damage involves the activation of a protein kinase ATM.[53]

(5) Chk2 is activated by ATM, and its activation involves dimerization and autophosphorylation.[54,55]

(6) ATM phosphorylates p53.[56,57]

(7) Chk2 also phosphorylates p53.[58]

(8) Phosphorylated p53 (p53-P) by ATM or Chk2 leads to an increase in the transcription of Mdm2.[59,60] Process no.8 can be divided into two detailed processes as follows (See **Fig. 1B**):

(8-1) p53-P transcribes *mdm2* mRNA after a time delay τ_1. The transcription rate of *mdm2* mRNA activated by p53-P depends on the concentration of p53-P at time t-τ_1. Delay time τ_1

corresponds to the transcription and splicing processes of the Mdm2 gene into Mdm2 mRNA. We estimate the transcriptional time delay τ_1 to be 30 min.[11]

(8-2) Mdm2 is translated from Mdm2 mRNA after a time delay τ_2. The rate of production of nuclear Mdm2 depends on the concentration of Mdm2 mRNA at time t-τ_2. Delay time τ_2 corresponds to the translocation of Mdm2 mRNA to the cytosol, the translation of Mdm2 mRNA into Mdm2 protein, and the transport of Mdm2 back to the nucleus to become nuclear Mdm2 at time *t*. So Mdm2(t-τ_2) is transported out of the nucleus, translated, and translocated back to the nucleus. The translational/translocational delay τ_2 is assumed to be 10 min.[11]

(9) Mdm2 targets p53 for the proteasome-mediated degradation.[61-63] Mdm2-binding prevents the transcriptional activity of p53. Although p53 is transcriptionally inactive when bound to Mdm2, the previous mathematical models assume that p53 degradation depends on the total Mdm2 levels regardless of whether Mdm2 is bound to p53 or not. In contrast, our model considers the p53·Mdm2 complex, which will be degraded by proteasome. Here we use the concept of the p53·Mdm2 complex as having both p53·Mdm2 and p53-P·Mdm2 for brevity. The binding interaction by Mdm2 can be divided into two (See **Fig. 1B**):

(9-1) Mdm2 binds with p53 to create the p53·Mdm2 complex and promotes its fast degradation.

(9-2) Mdm2 binds with p53-P to create the p53·Mdm2 complex and promotes its slow degradation.

It is known that the effective activity of Mdm2 experienced by p53 is higher than that experienced by p53-P. Therefore, the results on the control of mono- versus poly-ubiquitination of p53 by Mdm2,[64] suggest that p53 and p53-P may follow different degradation pathways, with p53 having about 5-fold higher degradation rate compared with p53-P.[11]

(10) ATM phosphorylates Mdm2 to enhance the degradation of Mdm2.[65-68]

(11) ARF binds to Mdm2 and enhances the degradation of Mdm2. ARF thus increases the levels of p53 indirectly.[39-43]

(12) p53-P negatively regulates ARF expression, creating another feedback loop through which p53 activity is modulated.[24,69-73] Although this negative regulation has been accepted to exist in some review papers,[24,72,73] it is not clear whether the interaction between p53 and ARF really exists or not. Therefore, we consider both cases where this interaction is included, and the opposite one where it is excluded, as shown in **Figure 2**.

(13) Wip1 is induced by p53-P.[74]

(14) Wip1 dephosphorylates p53-P.[75]

(15) Wip1 dephosphorylates Mdm2 to stabilize it.[76]

(16) Wip1 dephosphorylates ATM.[77]

(17) Wip1 dephosphorylates Chk2.[78,79]

The mathematical equations for our model are given as follows:

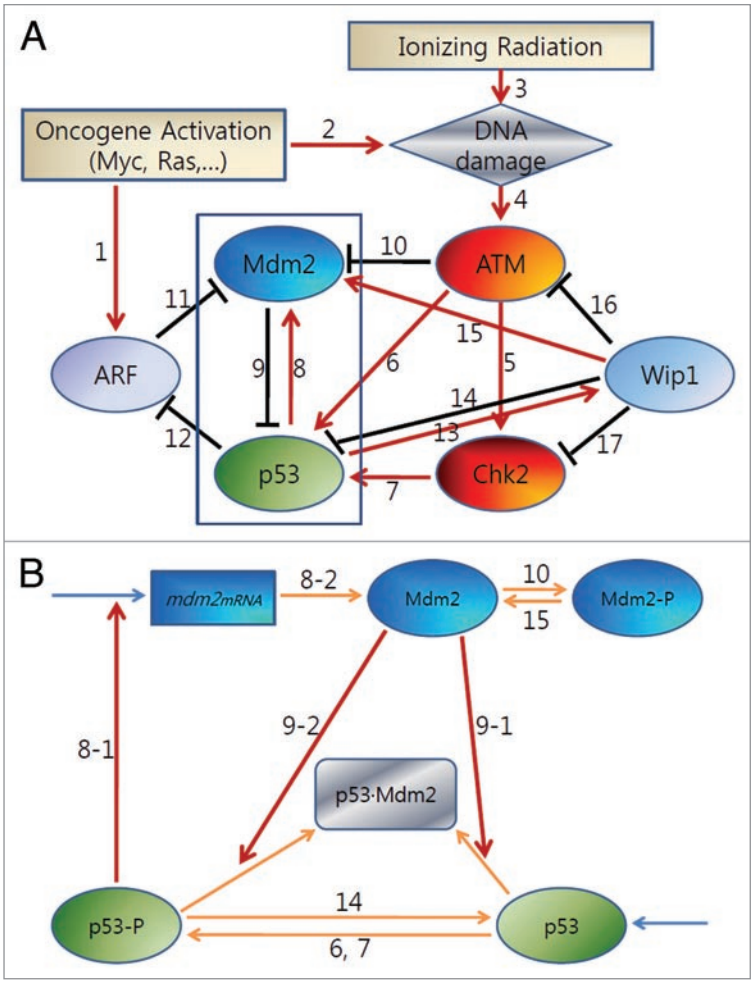

Figure 1. (A) Model for p53 dynamics. Each number in this figure denotes each interaction between two proteins. (B) Detailed figure for the part of no. 8 and 9 (blue box) in (A).

$$\frac{d[ARF]}{dt} = \alpha_1[oncogene] - \alpha_{12}[ARF][p53\text{-}P(t-\tau_{12})] - \gamma_{ARF}[ARF]$$

$$[DNA\ damage] = \alpha_2[oncogene] + \alpha_3[radiation]$$

$$\frac{d[ATM]}{dt} = \alpha_4[DNA\ damage] - \alpha_{16}[ATM][Wip1] - \gamma_{ATM}[ATM]$$

$$\frac{d[Chk2]}{dt} = \alpha_5[ATM] - \alpha_{17}[Chk2][Wip1] - \gamma_{Chk2}[Chk2]$$

$$\frac{d[p53]}{dt} = \beta_{p53} - \alpha_6[p53][ATM] - \alpha_7[p53][Chk2] - \alpha_{9-1}[p53]([Mdm2] - [p53\cdot Mdm2]) + \alpha_{14}[p53\text{-}P][Wip1] - \gamma_{p53}[p53]$$

$$\frac{d[p53\text{-}P]}{dt} = \alpha_6[p53][ATM] + \alpha_7[p53][Chk2] - \alpha_{9-2}[p53\text{-}P]([Mdm2] - [p53\cdot Mdm2]) - \alpha_{14}[p53\text{-}P][Wip1] - \gamma_{p53\text{-}P}[p53\text{-}P]$$

$$\frac{d[p53\cdot Mdm2]}{dt} = \alpha_{9-1}[p53]([Mdm2] - [p53\cdot Mdm2]) + \alpha_{9-2}[p53\text{-}P]([Mdm2] - [p53\cdot Mdm2]) - \gamma_{p53\cdot Mdm2}[p53\cdot Mdm2]$$

$$\frac{d[mdm2mRNA]}{dt} = \beta_{mdm2mRNA} + \alpha_{8-1}[p53\text{-}P(t-\tau_{8-1})] - \gamma_{mdm2mRNA}[mdm2mRNA]$$

$$\frac{d[Mdm2]}{dt} = \alpha_{8-2}[mdm2mRNA(t-\tau_{8-2})] - \alpha_{10}[Mdm2][ATM] - \alpha_{11}[Mdm2][ARF] + \alpha_{15}[Mdm2\text{-}P][Wip1] - \gamma_{Mdm2}[Mdm2]$$

$$\frac{d[Mdm2\text{-}P]}{dt} = \alpha_{10}[Mdm2][ATM] - \alpha_{15}[Mdm2\text{-}P][Wip1] - \gamma_{Mdm2\text{-}P}[Mdm2\text{-}P]$$

$$\frac{d[Wip1]}{dt} = \alpha_{13}[p53\text{-}P(t-\tau_{13})] - \gamma_{Wip1}[Wip1]$$

All the parameters were reasonably chosen to fit the theoretically and experimentally observed behaviors of p53 and other

Table 1. Parameters used in the mathematical model

Parameter	Meaning	Value	Unit
$\tau_{8\text{-}1}$	transcriptional time delay by p53	30	min
$\tau_{8\text{-}2}$	translational/translocational time delay by p53	10	min
τ_{12}	time delay of p53 inhibition to ARF	90	min
τ_{13}	time delay in Wip1 production	75	min
α_1	ARF production rate by oncogene activation	0.03	min^{-1}
α_2	DNA damage produced by oncogene activation	0.5	
α_3	DNA damage produced by ionizing radiation	0.5	$SU\ Gy^{-1}$
α_4	ATM activation rate by DNA damage	0.1	min^{-1}
α_5	Chk2 activation rate by phosphorylated ATM	0.03	min^{-1}
α_6	ATM-dependent phosphorylation rate of p53	0.6	$SU^{-1}\ min^{-1}$
α_7	Chk2-dependent phosphorylation rate of p53-P	0.5	$SU^{-1}\ min^{-1}$
$\alpha_{8\text{-}1}$	translation rate of *mdm2 mRNA* activated by p53-P	0.02	min^{-1}
$\alpha_{8\text{-}2}$	production rate of Mdm2	0.02	min^{-1}
$\alpha_{9\text{-}1}$	Mdm2-dependent fast degradation of p53	0.9	$SU^{-1}\ min^{-1}$
$\alpha_{9\text{-}2}$	Mdm2-dependent slow degradation of p53-P	0.2	$SU^{-1}\ min^{-1}$
α_{10}	ATM-dependent phosphorylation rate of Mdm2	0.015	$SU^{-1}\ min^{-1}$
α_{11}	Mdm2 degradation by ARF binding	0.05	$SU^{-1}\ min^{-1}$
α_{12}	negative regulation of ARF by p53-P	0.03	$SU^{-1}\ min^{-1}$
α_{13}	Wip1 induction by p53-P	0.04	$SU^{-1}\ min^{-1}$
α_{14}	p53-P dephosphorylation by Wip1	0.05	$SU^{-1}\ min^{-1}$
α_{15}	Mdm2-P dephosphorylation by Wip1	0.02	$SU^{-1}\ min^{-1}$
α_{16}	ATM dephosphorylation by Wip1	0.2	$SU^{-1}\ min^{-1}$
α_{17}	Chk2 dephosphorylation by Wip1	0.03	$SU^{-1}\ min^{-1}$
β_{p53}	p53 production rate	0.06	$SU\ min^{-1}$
$\beta_{mdm2mRNA}$	*mdm2 mRNA* production rate	0.02	$SU\ min^{-1}$
γ_{ARF}	ARF degradation rate	0.0027	min^{-1}
γ_{ATM}	ATM degradation rate	0.015	min^{-1}
γ_{Chk2}	Chk2 degradation rate	0.015	min^{-1}
γ_{p53}	p53 degradation rate	0.03	min^{-1}
$\gamma_{p53\text{-}P}$	p53-P degradation rate	0.015	min^{-1}
$\gamma_{p53\cdot Mdm2}$	p53·Mdm2 proteosomal degradation rate	0.06	min^{-1}
$\gamma_{mdm2mRNA}$	*mdm2 mRNA* degradation rate	0.02	min^{-1}
γ_{Mdm2}	Mdm2 degradation rate	0.015	min^{-1}
$\gamma_{Mdm2\text{-}P}$	Mdm2-P degradation rate	0.015	min^{-1}
γ_{Wip1}	Wip1 degradation rate	0.05	min^{-1}

Here, SU means Simulation Unit. Notice that all values of initial conditions of 9 proteins during all simulations are chosen as zero (i.e., [*p53*(0)] = [*p53-P*(0)] = … = 0) but the initial value of *mdm2mRNA* is chosen as 0.1 (i.e., [*mdm2mRNA*(0)] = 0.1).

proteins. The values for all the parameters used in this model are given in **Table 1**. Here, the 17 terms with parameters α_i (i = 1, 2, …, 17) correspond to the 17 interactions explained above. The β_{p53} and $\beta_{mdm2mRNA}$ denote the production rate of p53 and mdm2 mRNA, respectively. Each γ_j (j = ARF, ATM, …) term denotes the degradation rate of each considered protein or mRNA. Each τ_k (k = 8-1, 8-2, 12, 13) term corresponds to the time delay between p53 and other protein (Mdm2, Wip1 or ARF). We chose all parameter values from the established results of previous experimental and theoretical papers. For example, γ_{ARF} was selected as

0.0027 min^{-1}, and it was derived from the half-life of ARF at about 6 h.[80] In this model, we did not divide inactive ATM (Chk2) by active phosphorylated ATM (Chk2), that is, ATM-P (Chk2-P) for brevity. We carried out numerical simulations using Wolfram Mathematica 7.0 (Wolfram Research, Inc.).

In our mathematical model, we did not include both the Michaelis-Menten and the Hill equation. These two equations depend on some assumptions that may not be applicable to our p53 system. To satisfy the Michaelis-Menten equation, there must be very little enzymes compared with the substrate (i.e., [*E*] << [*S*]).[81] Therefore, to consider ATM or Mdm2 as an enzyme of substrate p53, as some previous papers have assumed,[11-14] we must accept that [ATM(Mdm2)] << [p53] in our model, which is not true. In both equations, the concentration of the substrate-bound enzyme [(n*ES*), where n is the Hill coefficient; the Michaelis-Menten equation corresponds to the case of n = 1] does not change with time (the quasi-steady-state approximation).[82] However, the rate of change of [n*ES*] cannot be zero, as we can easily check from the amount of [p53·Mdm2]. Through careful simulations, we easily determined that the overall oscillatory behaviors of all proteins were not changed by the insertions of the Michaelis-Menten or Hill equation to some terms, if the Michaelis or Hill dissociation constant was not too large.

Results and Discussions

By using our simulations, we primarily want to check whether interaction no.12 (negative regulation of ARF by p53-P) exists in real situations or not. We thus considered the three cases: one case where interaction no.12 with nonzero τ_{12} is included, one case in which interaction no.12 contains zero τ_{12}, and one case without interaction no.12. In such cases, the dynamics of p53 occur by oncogene activation in the case with no ionizing radiation, are shown in **Figure 2**. In **Figure 2A**, the pulses of all proteins maintain their heights and widths when the time delay τ_{12} is prolonged (>1 h). ARF and DNA damage are originated by the activated oncogenes, and ATM, Chk2, p53, Wip1 and Mdm2 follow them one after the other. After their activations, their amounts decrease by negative feedbacks and then increase again, making their oscillations occur within a specific period and time delay. Thus, our model shows that oncogene activation and ARF with nonzero τ_{12} have major roles in producing and maintaining sustained oscillations of p53 and other proteins. However, the pulses of all proteins when τ_{12} is fixed as zero show damped oscillations in **Figure 2B**. Thus, we presume that the time delay τ_{12} is an important factor in extending the damping of pulses. Furthermore, when we exclude interaction no.12 from our theoretical model, the oscillatory behaviors of all proteins become unsustainable, as shown in **Figure 2C**. This is due to the excessive amount of ARF without any inhibition by other proteins. Therefore, there must be a negative regulation of ARF by p53-P to sustain their oscillations. Thus, we surmise that in real experimental situations, p53-P may negatively regulate ARF in situations with sufficiently prolonged time delay through direct interaction between them or an indirect one mediated by other proteins. This is a novel theoretical estimation given by

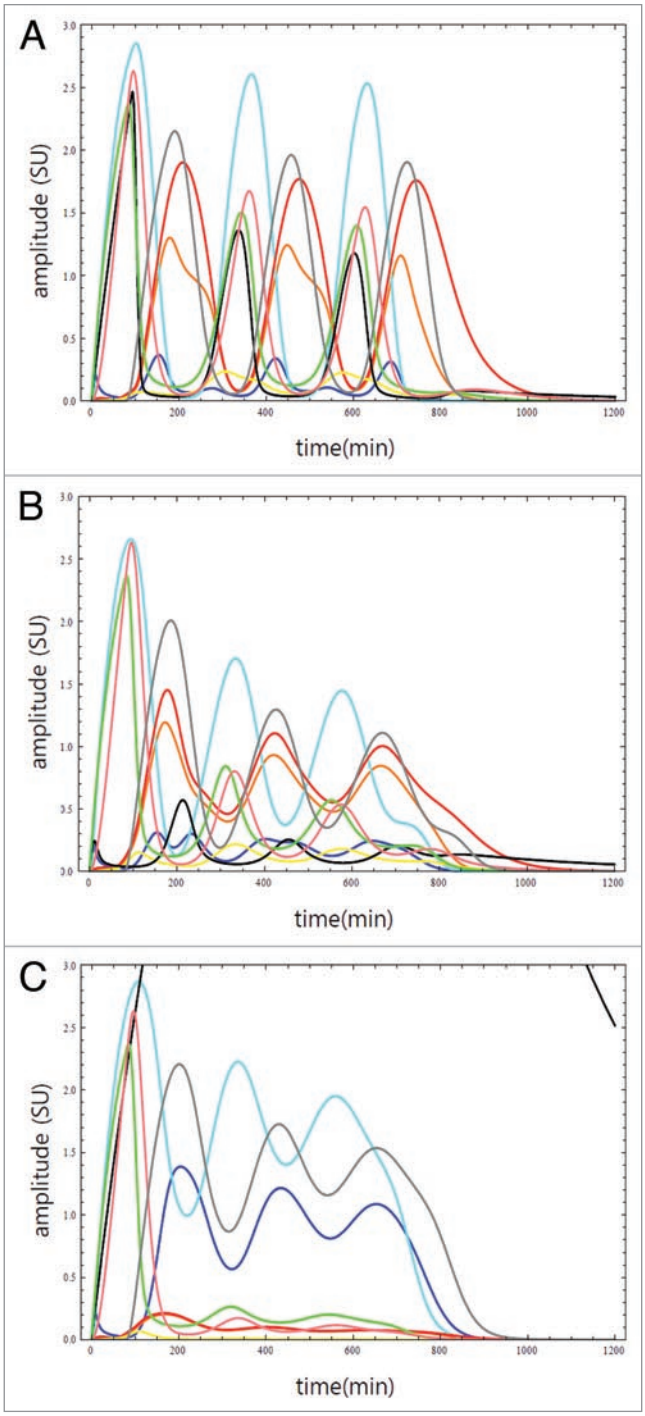

Figure 2. (A) The oscillations of proteins generated by an amount of activated oncogene given as 9.0 SU (Simulation Unit) with no ionizing radiation. Values for all parameters used are given in Table 1. (B) The damped oscillatory behaviors of proteins generated under the same conditions with (A), except at τ_{12} = 0. (C) The unsustained oscillations of proteins that took place when interaction no.12 was not included. Each colored line corresponds to each protein: ARF (Black), ATM (Green), Chk2 (Pink), p53 (Blue), p53-P (Cyan), p53·Mdm2 (Orange), Wip1 (Gray), Mdm2 (Red) and Mdm2-P (Yellow).

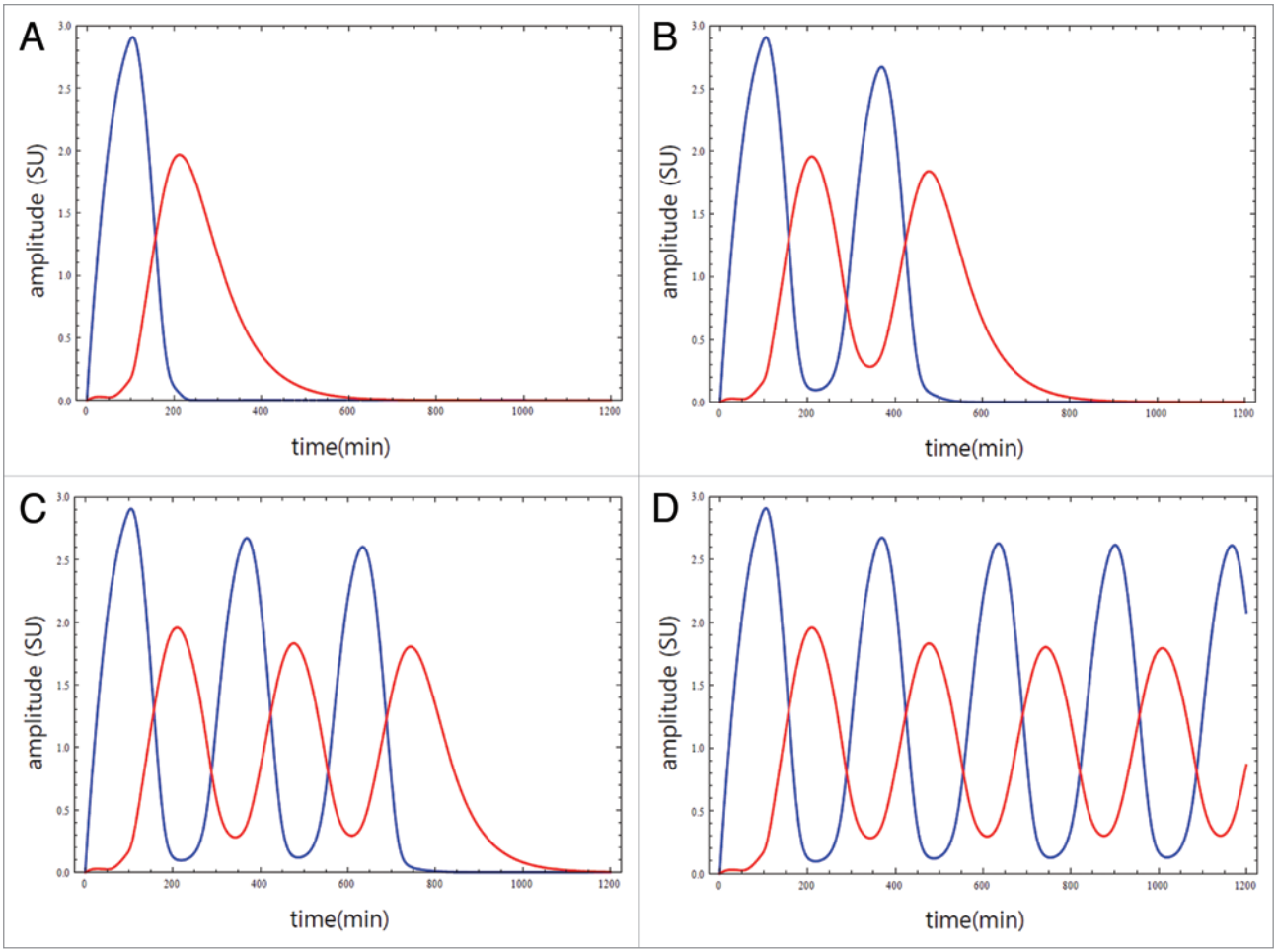

Figure 3. The oscillatory behaviors of total p53 (= p53 + p53 - P) (blue) and total Mdm2 (= Mdm2 + Mdm2 - P) (red) generated by activated oncogene with a value of 2.6 (A), 6.0 (B), 9.0 (C) and 20.0 SU (D). The height of the first pulse was 2.92 and that of the second pulse was 2.65, irrespective of the values of activated oncogene.

our model, which may be validated by experiments in the near future.

According to the previous experimental work of Lahav et al.[19] p53 has a "digital" behavior: The height and width of p53 pulses in single cells do not depend on the level of the input damage signal, but rather, they preserve their own values. Instead, the number of p53 pulses depends on the level of the input damage signal. As our model is made to account for the p53 dynamics in single cells, our model should show the digital behavior. The figures in **Figure 3** present the oscillatory dynamics of p53 and Mdm2 dependent on a given value of activated oncogene in the absence of ionizing radiation. Four figures in **Figure 3** clearly show that the number of p53 pulses increases in proportion to the input value of the activated oncogene. Furthermore, when we compare the value of the p53 pulse width (height) of **Figure 3A** with one of the p53 first pulse widths (heights) of **Figure 3B–D**, we can easily confirm that our results satisfy the digital behavior of p53 pulse. When we compare the value of the p53 second pulse width (height) of **Figure 3B** with one of the p53 second pulse widths (heights) of **Figure 3C and D**, the digital behavior is again demonstrated. Therefore, we confirm that our model maintains the digital behavior of p53 pulse in single cells well. The width of p53 pulses in our simulations is directly proportional to the both values of $\tau_{8\text{-}1}$ (transcriptional time delay by p53) and $\tau_{8\text{-}2}$ (translational/translocational time delay by p53). Therefore, the large widths (~350 min) of the first and second pulses of p53 in **Figure 3B and C** of Lahav et al.[19] may have come from the extra time-delaying processes—such as interactions with other proteins—beyond the transcriptional, translational and translocational activities of p53-P considered in our simulations.

In **Figure 4A and B**, we illustrate the graphs of p53, ATM, and Chk2 together to compare them with the **Figure 1C** of Batchelor et al.[16] which was obtained by performing immunoblots of p53, ATM and Chk2 in response to γ-irradiation. Batchelor et al.[16] explained the repression of ATM and Chk2 levels between their pulses through the presence of another negative feedback mechanism connecting p53 with ATM and Chk2 in the form of two kinases signaling the presence of DNA damage by ionizing radiation, thus introducing a negative feedback loop between p53 and Wip1, the inhibitor of ATM and Chk2. By accepting the same negative feedback mechanism between p53 and Wip1 in our model, we can obtain qualitatively similar pulsatile dynamics of

p53, ATM and Chk2, as shown in **Figure 4A and B**. In **Figure 4B**, the case which considers ionizing radiation as the only single stimulus with no oncogene activation, the pattern of the heights of Chk2 pulses is similar to the results obtained by Batchelor et al. (**Fig. 1C** of ref. 16) measured under the response to γ-irradiation. Furthermore, the ratio of the second p53 pulse height to the first one in **Figure 4B** is about 0.8, which is nearly the same value as that in **Figure 3D and E** of Lahav et al.[19] Thus, this may be considered as one evidence that the p53 dynamics of Lahav et al.[19] comes only from the γ-irradiation without extra oncogene activation.

Now we turn our attention to determining the most optimal therapeutic strategy that may help stabilize and activate p53. Any optimal therapeutic strategy can be evaluated by the amount of responses of p53 pulses when a correspondent parameter is varied (increased or decreased) slightly. For example, if we want to examine the insertion effect of small-molecule inhibitors of the p53-Mdm2 interaction such as Nutlins, we can check it by measuring the sensitivity of p53 pulses when both parameters $\alpha_{9\text{-}1}$ (Mdm2-dependent fast degradation of p53) and $\alpha_{9\text{-}2}$ (Mdm2-dependent slow degradation of p53-P) are decreased. Sensitivity of any p53 pulse can be defined by the variation of its amplitude (height), period (width) or area surrounded by the pulse line and time axis (∝ height·width). As the amplitude or period of p53 pulse does not vary sensitively compared with its area, we use the area as a measure for sensitivity of the p53 pulses. The area can be calculated numerically using the definite integral.

When we investigated the sensitivity of p53 pulses with respect to the variations of all α and β parameters in **Table 1**, we found that it was highly dependent on the variations of the following parameters: β_{p53} (p53 production rate), $\alpha_{8\text{-}1}$ (translation rate of Mdm2mRNA activated by p53-P) or $\alpha_{8\text{-}2}$ (production rate of Mdm2), $\alpha_{9\text{-}1}$ and $\alpha_{9\text{-}2}$ (Mdm2-dependent degradation of p53 and p53-P), α_1 (ARF production rate by oncogene activation) or α_{11} (Mdm2 degradation by ARF binding). The other parameters aside from the ones mentioned vary the shapes and areas of p53 pulses only slightly. In the Introduction, we considered the four possible strategies for therapeutic targeting of the p53 pathway, which correspond to the following parameters:

(1) Promoting the production of wild-type p53 can be conducted by increasing β_{p53}

(2) Decreasing levels of Mdm2 can be achieved by decreasing $\alpha_{8\text{-}1}$ or $\alpha_{8\text{-}2}$

(3) Releasing p53 from Mdm2 by use of inhibitors of the p53-Mdm2 interaction such as Nutlins can be conducted by decreasing both $\alpha_{9\text{-}1}$ and $\alpha_{9\text{-}2}$

(4) Binding to Mdm2 and enhancing the degradation of Mdm2 by ARF can be attained by increasing α_1 or α_{11}.

Now, let us determine which strategy is the most optimal for therapeutic targeting.

Figure 5 shows the sensitivity of p53 pulses under several kinds of variations of β_{p53}, $\alpha_{8\text{-}1}$ or $\alpha_{8\text{-}2}$, both $\alpha_{9\text{-}1}$ and $\alpha_{9\text{-}2}$, and α_1 or α_{11} in the case of no ionizing radiation. It is obvious that p53 pulses, especially their amplitudes, change most drastically when the parameter β_{p53} is varied, as shown in **Figure 5A and Table 2**. Therefore, the intentional insertion or increase of p53 production

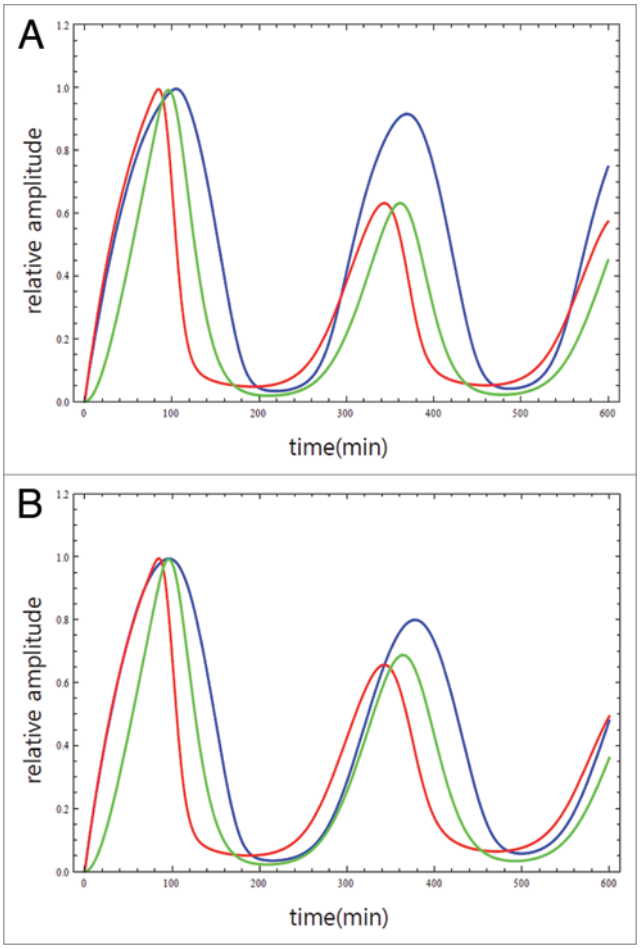

Figure 4. The oscillatory behaviors of total p53 (= p53 + p53 - P) (blue), ATM (red) and Chk2 (green) generated by activated oncogene with a value of 9.0 SU without ionizing radiation (A) and by ionizing radiation with a value of 9.0 Gy when there is no oncogene activation (B). The amplitudes of three proteins are normalized in units relative to the first peak intensity. The ratio of the second pulse height to the first one in (B) is nearly 0.8.

rate may be the best strategy for therapeutic targeting of the p53 pathway. However, previous trials of p53 gene therapy have not shown noteworthy results in real clinical cases (reviewed in ref. 83). Thus, it is necessary to search for another good candidate for therapeutic targeting. In the case of decreasing the amount of Mdm2 by controlling $\alpha_{8\text{-}1}$ or $\alpha_{8\text{-}2}$, the areas of p53 pulses increase in proportion to the decrease of $\alpha_{8\text{-}1}$ or $\alpha_{8\text{-}2}$ (see **Table 2**), but the shape of pulses are far from sustained oscillations, as in **Figure 5B**, the conditions of which are not suitable to the interests of this paper.

Thus, we need other therapeutic strategies that not only can increase the areas of pulses, which guarantees the activation of p53, but can also maintain the robustness of their oscillatory behaviors, which guarantees the stabilization of p53. Strategies 3, the releasing of p53 from Mdm2 using the inhibitors of the p53-Mdm2 interaction such as Nutlins, and 4, binding to Mdm2 and enhancing the degradation of Mdm2 by ARF, seem to be good candidates that satisfy both conditions. When we compare the

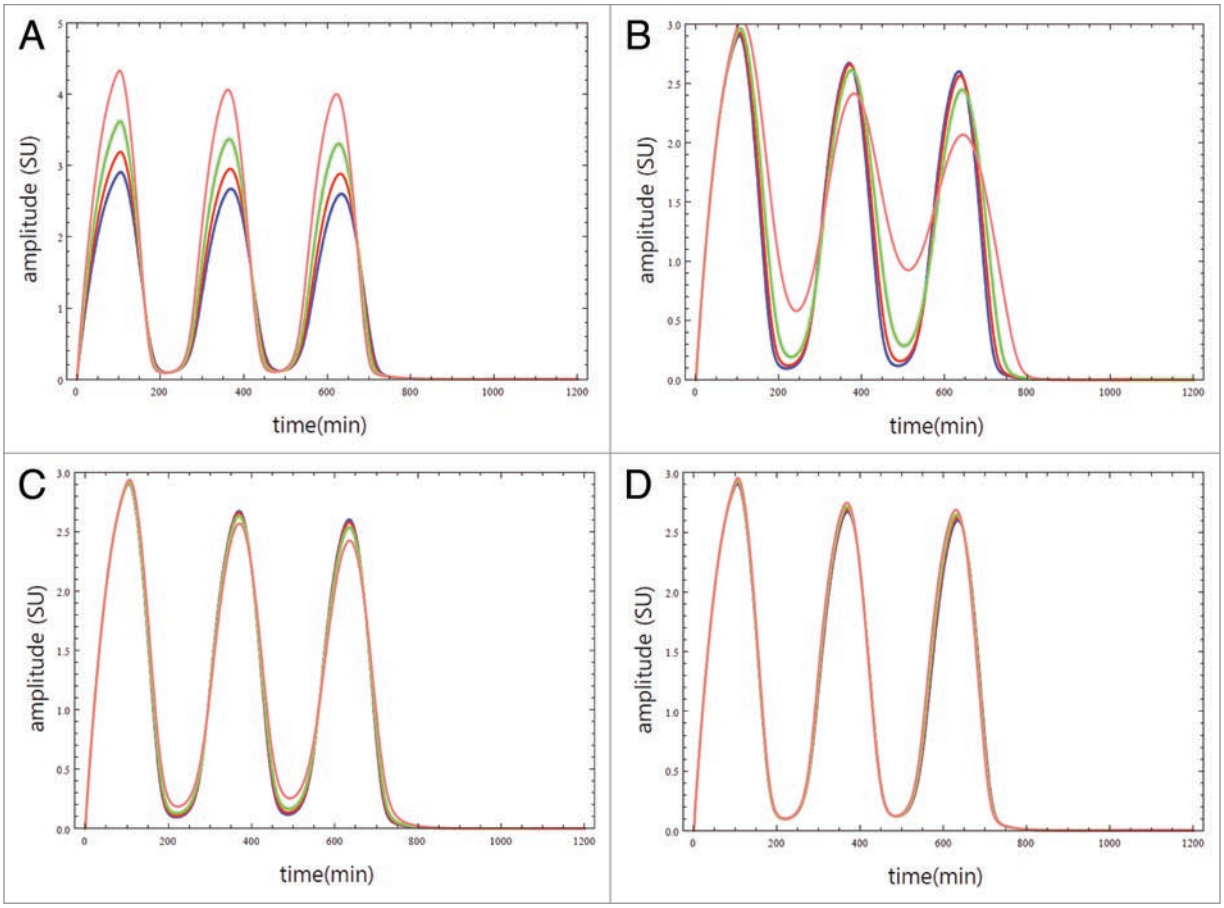

Figure 5. The sensitivity of total p53 (= p53 + p53 - P) pulses generated by activated oncogene with a value of 9.0 SU with respect to the variations of β_{p53} (A), $\alpha_{8\text{-}2}$ (B), both $\alpha_{9\text{-}1}$ and $\alpha_{9\text{-}2}$ (C), and α_{1} or α_{11} (D). Blue lines are the results obtained under original parameter values of Table 1; the red lines are attained from 10% variations of the concerned parameters; the green lines from 25% variations; and the pink lines from 50% variations.

Table 2. The sizes of total p53 (= p53 + p53 - P) pulses generated under the condition of figure 5 depending on the variation of concerned parameters

Parameter	Original value	10% variation	25% variation	50% variation
β_{p53}	979.15	1063.00	1188.09	1395.09
$\alpha_{8\text{-}1}$	979.15	1011.43	1071.16	1233.69
$\alpha_{8\text{-}2}$	979.15	1011.66	1071.80	1235.24
$\alpha_{9\text{-}1}$ and $\alpha_{9\text{-}2}$	979.15	983.43	991.84	1015.87
α_{1} or α_{11}	979.15	986.07	995.54	1009.47

areas of p53 pulses with each other, the area in strategy 4 is generally larger than in strategy 3, as shown in **Table 2**. Furthermore, as the amplitude of pulses in strategy 3 decreases more rapidly with time, we can say that the shape of p53 pulses in strategy 4 shows more robust oscillations, as presented in **Figure 5C and D**. Therefore, according to our deterministic theoretical model, strategy 4, which is Mdm2 degradation by ARF binding, can be accepted as the optimal one for therapeutic targeting because it guarantees both the activation and stabilization of p53. Although it does not increase the level of p53 directly like strategy 1 but rather inhibits Mdm2, which is the inhibitor of p53, it still eventually increases p53 indirectly.

Conclusion

We introduced an expanded theoretical model for p53 dynamics which include the oncogene-mediated activation of ARF. Through the numerical calculations, we theoretically validated several important features of the p53 pathways observed in previous studies.

Primarily, we stressed the role of the oncogene-mediated activation of ARF as an important factor for the activation and stabilization of p53 in the case of no ionizing radiation. ARF plays a role in the inhibition of Mdm2, such that ARF increases the level in p53 indirectly, and this has been accepted as a crucial pathway

for tumor suppression.[20-22] Our theoretical model showed the role of ARF as well as that of ATM/Chk2 in aiding the p53-Mdm2 loop to drive sustained oscillations of p53.

Secondly, we revealed the importance of time delays in negative feedback loops to guarantee sustained p53 oscillations by controlling the values of time delays, which we used in our model. All the robust oscillations of p53, Mdm2, ATM, Chk2 and Wip1 occurred due to the existence of time delays, as shown in **Figures 2 and 4**. We showed that interaction no.12 (negative regulation of ARF by p53-P) should have its own time delay for sustaining oscillations of p53.

Thirdly, as shown in **Figure 3**, we clearly confirm the digital behavior of the p53 pulses, first described in the seminal work of Lahav et al.[19] by showing that their heights and widths in single cells can be kept constant irrespective of the input amount of oncogene activation and that instead the number of pulses depend on it.

Furthermore, we strongly propose that binding to Mdm2 and enhancing the degradation of Mdm2 by ARF can be considered as one of the most optimal strategies for therapeutic targeting, which guarantees the activation and stabilization of p53. So far, there has been much effort to develop new techniques for promoting the production of wild-type p53,[28-32] and for releasing p53 from Mdm2 using the inhibitors of the p53-Mdm2 interaction such as Nutlins.[35,36] Our model proposes that ARF, as an inhibitor of Mdm2, is also useful as a therapeutic target for the activation and stabilization of p53.

The dynamics of p53 has been considered as an important topic both in pure scientific research and in therapeutic, pharmaceutical and pharmacological purposes during the last 30 years.[84] We expect that our theoretical approach will be refined and expanded further by coming up with more experimental evidence, thus having more values in the near future.

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