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ABSTRACT

The NF- κ B family of transcription factors is essential for promoting cell proliferation and preventing cell apoptosis. We have previously shown that Andrographolide (Andro) isolated from an herbal plant, *Andrographis paniculata*, covalently modifies reduced cysteine⁶² in the oligonucleotide binding pocket of p50 for inhibition of NF- κ B activation. Here we report that Andro, but not its inactive structural analog 4H-Andro, potently suppressed squamous cell carcinogenesis induced by 7,12-dimethyl-1,2-benzanthracene (DMBA) in the hamster model of cheek buccal pouch. Compared with 4H-Andro, Andro reduced phosphorylation of p65 (Ser536) and I κ B α (Ser32/36) for inhibiting aberrant NF- κ B activation, suppressed c-Myc and cyclin D1 expression and attenuated neoplastic cell proliferation, promoted cancerous cell apoptosis, and mitigated tumor-induced angiogenesis. Consistently, Andro retarded growth, decreased proliferation, and promoted apoptosis of Tb cells, a human tongue squamous cell carcinoma cell line, in time- and dose-dependent manners, with concomitant reduction of the expression of NF- κ B targeting molecules *in vitro*. Our results thus demonstrate that NF- κ B activation plays important roles in the pathogenesis of chemically induced squamous cell carcinoma. By inhibition of aberrant NF- κ B activation, Andro treats chemically induced oral squamous cell carcinogenesis.

KEY WORDS: NF- κ B activation, chemically induced carcinogenesis, Andrographolide, squamous cell carcinoma, proliferation, hamster buccal pouch.

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Andrographolide Inhibits Oral Squamous Cell Carcinogenesis through NF- κ B Inactivation

INTRODUCTION

Oral squamous cell carcinoma is the sixth most common neoplasm worldwide, with more than half a million new cases being diagnosed annually (Parkin *et al.*, 2005; Allen *et al.*, 2007), particularly in certain areas of Asia, the Pacific Islands, Europe, and Brazil, where it constitutes up to one-quarter of all human cancers (Funk *et al.*, 2002; Jemal *et al.*, 2004). Unfortunately, survival of oral squamous cell carcinoma patients has not been significantly improved, despite recent advances in chemotherapy and radiotherapy (Davies and Welch, 2006). It is therefore imperative that we understand the underlying molecular mechanisms during the pathogenesis of oral squamous cell carcinoma and discover novel therapeutic targets for the prevention and treatment of this devastating disease.

The nuclear factor- κ B (NF- κ B) family of transcription factors includes p105/p50 (NF- κ B1), p100/p52 (NF- κ B2), p65 (RelA), RelB, and c-Rel, among which the p50/p65 heterodimer is most abundant (Li and Verma, 2002; Hayden and Ghosh, 2008). The p50/p65 heterodimer is localized in the cytoplasm, where it binds to I κ B inhibitory proteins, including I κ B α , I κ B β , and I κ B ϵ . Upon stimulation, I κ B proteins are rapidly phosphorylated by I- κ B kinase α and β (IKK α and IKK β) and are degraded *via* the ubiquitin-proteasome pathway (Shishodia and Aggarwal, 2004). Degradation of I κ B proteins exposes the nuclear localization signals on the p50/p65 heterodimer, which then translocates to the nucleus for transcriptional up-regulation of a variety of downstream genes that govern innate immunity, inflammation, cell growth, and apoptosis (Gupta *et al.*, 2010). Notably, p65 phosphorylation by both IKK α and β is critically required for its liberation from the IKK complex during NF- κ B activation (Sizemore *et al.*, 2002).

Andrographolide (Andro) is a diterpenoid lactone isolated from a plant called *Andrographis paniculata*, which has been used widely as a regimen of traditional herbal medicine for amelioration of sore throat, diarrhea, and other inflammatory disorders in Asian countries for more than two millennia. Recently, Andro has been shown to inhibit the *in vitro* cell growth of hepatocellular carcinoma (Zhou *et al.*, 2006), cervical carcinoma (Rajagopal *et al.*, 2003), prostatic adenocarcinoma (Kim *et al.*, 2005), and colorectal carcinoma (Kumar *et al.*, 2004). Mechanistically, Andro is known to induce apoptosis of human cancer cells *via* the death-receptor-mediated apoptotic pathway (Rajagopal *et al.*, 2003). It also sensitizes tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in TRAIL-resistant human cancer cells (Zhou *et al.*, 2008). In addition, Andro affects the activity

of CDK P^{cdc2} and induces the expression of cell-cycle inhibitor P27 for restraining tumor cells at G₀/G₁ (Zhou *et al.*, 2006). By suppressing the ERK1/2 pathway, it reportedly induces TNF- α synthesis and stimulates production of cytotoxic T-lymphocytes for the inhibition of tumor growth *in vivo* (Qin *et al.*, 2006). Furthermore, Andro inhibits tumor-induced angiogenesis by decreasing the expression of vascular endothelial growth factor (VEGF), nitric oxide (NO), and various inflammatory cytokines and chemokines while increasing the expression of endogenous anti-angiogenesis factors, such as interleukin (IL)-2 and tissue inhibitors of metalloproteinase-1 (TIMP-1; Sheeja *et al.*, 2007). However, the biochemical mechanisms of how Andro inhibits cancers are still largely unknown. Notably, many chemically diverse substances from natural sources, especially foods, have been shown to target various steps in carcinogenesis, including survival, proliferation, apoptosis, angiogenesis, invasion, and metastasis of tumor cells (Gupta *et al.*, 2010).

In previous studies, we have shown that Andro covalently modifies reduced cysteine⁶² in the oligonucleotide binding pocket of p50 for inhibition of NF- κ B activation in inflammation (Xia *et al.*, 2004), arterial restenosis (Wang *et al.*, 2007), and deep vein thrombosis (Li *et al.*, 2009). Importantly, aberrant NF- κ B activation, which critically mediates cell proliferation and prevents cell apoptosis (Deveraux *et al.*, 1998), has been detected in a variety of human cancers, such as prostate carcinoma (Paule *et al.*, 2007), esophageal carcinoma (Yamada *et al.*, 2007), breast carcinoma (Ahmed *et al.*, 2006), intestinal carcinoma (Stark *et al.*, 2007), and oral carcinoma (Allen *et al.*, 2007; Garg *et al.*, 2008). Given the observed inhibitory activity of Andro on the NF- κ B signal transduction pathway, we speculated whether Andro could inhibit carcinogenesis through targeting p50 and consequently inactivating NF- κ B signaling. In this study, we tested this hypothesis using the hamster buccal pouch model of DMBA-induced squamous cell carcinoma and the human cell line of tongue squamous cell carcinoma Tb cells.

MATERIALS & METHODS

For complete information, please see the online Appendix.

RESULTS

NF- κ B Activation in Squamous Cell Carcinoma

Phosphorylation of p65 (Ser536) and I κ B α (Ser32/36) reportedly indicates NF- κ B activation, while c-Myc and cyclin D1 are well-known NF- κ B downstream targeting molecules (Li and Verma, 2002; Zhang *et al.*, 2005; Allen *et al.*, 2007; Hayden and Ghosh, 2008). To determine whether chemically induced oral squamous cell carcinogenesis could elicit aberrant NF- κ B activation, we examined total p65, p65 (Ser536), and I κ B α (Ser32/36) phosphorylation and c-Myc and cyclin D1 expression at various stages of DMBA-induced squamous cell carcinogenesis in the hamster buccal pouch model. According to the guidelines of WHO classification, tissue samples stained with H&E were classified into the stages of normal mucosa, hyperplasia, dysplasia, carcinoma *in situ*, and carcinoma (Fig. 1 and Appendix Table). Total p65,

phosphorylated p65 (Ser536), and I κ B α (Ser32/36) were not detected at the stages of normal mucosa and hyperplasia. However, total p65, phosphorylated p65 (Ser536), and I κ B α (Ser32/36) were clearly visible at the stage of dysplasia, further increased at the stage of carcinoma *in situ*, and peaked at the stage of carcinoma. Notably, phosphorylated p65 (Ser536) was restricted in the cytoplasm at stages of dysplasia and carcinoma *in situ*, whereas it was localized in both the cytoplasm and nucleus at the stage of carcinoma. In contrast, phosphorylated I κ B α (Ser32/36) was distributed exclusively in the cytoplasm. Consistently, the expression of cytoplasmic and nuclear c-Myc and nuclear cyclin D1 was not detected until the stage of hyperplasia, and gradually intensified at the stages of dysplasia and carcinoma *in situ*, finally peaking at the stage of carcinoma. These results clearly indicated that DMBA-induced oral squamous cell carcinogenesis triggers aberrant NF- κ B activation, especially in the pathological stages of dysplasia, carcinoma *in situ*, and carcinoma.

Andro Inhibits Oral Squamous Cell Carcinoma

We have previously shown that Andro covalently modifies reduced cysteine⁶² in the κ B binding pocket of p50, which drastically diminishes NF- κ B activation (Xia *et al.*, 2004). To elucidate whether NF- κ B activation critically participates in the pathogenesis of chemically induced squamous cell carcinoma, we tested whether Andro could inhibit DMBA-induced oral squamous cell carcinogenesis. Intraperitoneal treatment with Andro, but not saline (blank) or 4H-Andro (an inactive structural analog), drastically switched the appearance of almost all tumors from papillary protuberance (ulcerative, erosive, reddish, and fragile with micro-hemorrhage) into smooth, pale, and encapsulated tissue masses, with concomitant amelioration of edema and hyperemia in the neighboring tissues around cancerous lesions (Fig. 2A, left panel). Most importantly, Andro, but not 4H-Andro, potently reduced tumor volumes (Fig. 2A, right panel). Notably, no significant differences in the infiltrated leukocytes, including neutrophils and macrophages, were detected in the serial sections of tumor samples that had been treated with Andro, saline, and 4H-Andro (Fig. 2B), thus eliminating the possibility that Andro might affect leukocyte deposition within these cancerous tissues. These findings attested to the functional significance of NF- κ B activation during the pathogenesis of DMBA-induced oral squamous cell carcinoma. Consequently, Andro, but not 4H-Andro, decreased BrdU staining of DNA incorporation (Fig. 2C) and increased TUNEL staining of DNA fragmentation (Fig. 2D) of neoplastic tissues, which were markers for cell proliferation and apoptosis, respectively. Using the vWF antigen as a marker for vascular endothelial cells, we found that Andro, but not 4H-Andro, also diminished the formation of neovasculatures within the malignant tissues (Fig. 2E).

Andro Suppresses Aberrant NF- κ B Activation

We next tested whether Andro indeed prevented NF- κ B activation of oral squamous cell carcinoma through targeting p50. As predicted, Andro significantly reduced the expression of total

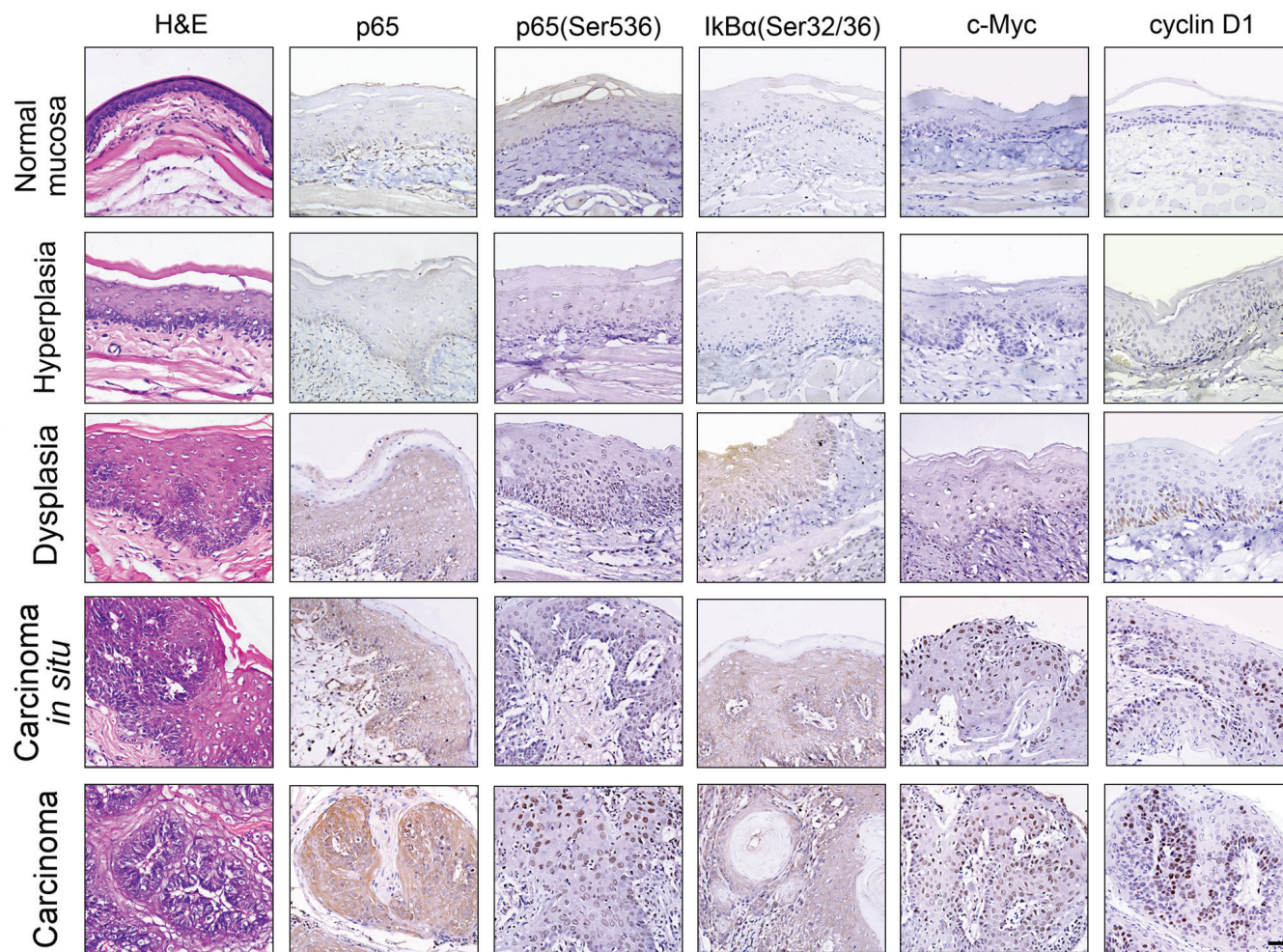


Figure 1. NF- κ B activation during chemically induced squamous cell carcinogenesis. The H&E staining and the immunohistochemical staining of total p65, phosphorylated p65 [Ser536], and I κ B α (pSer32/36) or c-Myc and cyclin D1 at the stages of normal, hyperplasia, dysplasia, carcinoma *in situ*, and carcinoma in DMBA-induced squamous cell carcinogenesis of the hamster buccal pouch. Results are representatives of at least 3 tissue samples from more than 3 hamsters for each group. Scale bars, 50 μ m.

p65 (Fig. 3A), phosphorylated p65 (Ser536) (Fig. 3B), I κ B α (Ser32/36) (Fig. 3C), c-Myc (Fig. 3D), and cyclin D1 (Fig. 3E) in tumor tissues as compared with 4H-Andro. These results convincingly demonstrate that Andro inhibits aberrant NF- κ B activation of chemically induced oral squamous cell carcinoma. Analysis of these data, taken together, suggests that Andro exerts its anti-cancer action by attenuating NF- κ B activation, suppressing tumor cell proliferation, promoting tumor cell apoptosis, and preventing tumor-induced angiogenesis through targeting NF- κ B transcription factor p50.

Effects of Andro on NF- κ B Activation and Survival of Tb Cells

In an effort to correlate our findings obtained from using the hamster model of chemically induced squamous cell carcinogenesis with human cancers, we tested the actions of Andro on human tongue squamous cell carcinoma Tb cells. Indeed, treat-

ment with Andro, but not DMSO, reduced the expression of total p65, phosphorylated p65 (Ser536), c-Myc, and cyclin D1 in a concentration-dependent manner (Fig. 4A). Compared with DMSO, Andro also inhibited growth and proliferation of Tb cells while inducing apoptosis in a dose- and time-dependent manner (Figs. 4B-4E). Our results thus provide evidence for the inhibition of growth and the promotion of apoptosis of human squamous cell carcinoma through inactivation of NF- κ B transcription factor p50.

DISCUSSION

Chemical induction of epithelial carcinogenesis in the buccal pouch of the Syrian hamster is a multistage model, which reiterates many features observed in human oral squamous cell carcinoma. This model has been widely used to test the efficacy of therapeutic agents, due mainly to easy accessibility for examination and follow-up of the oral lesions (Salley, 1954;

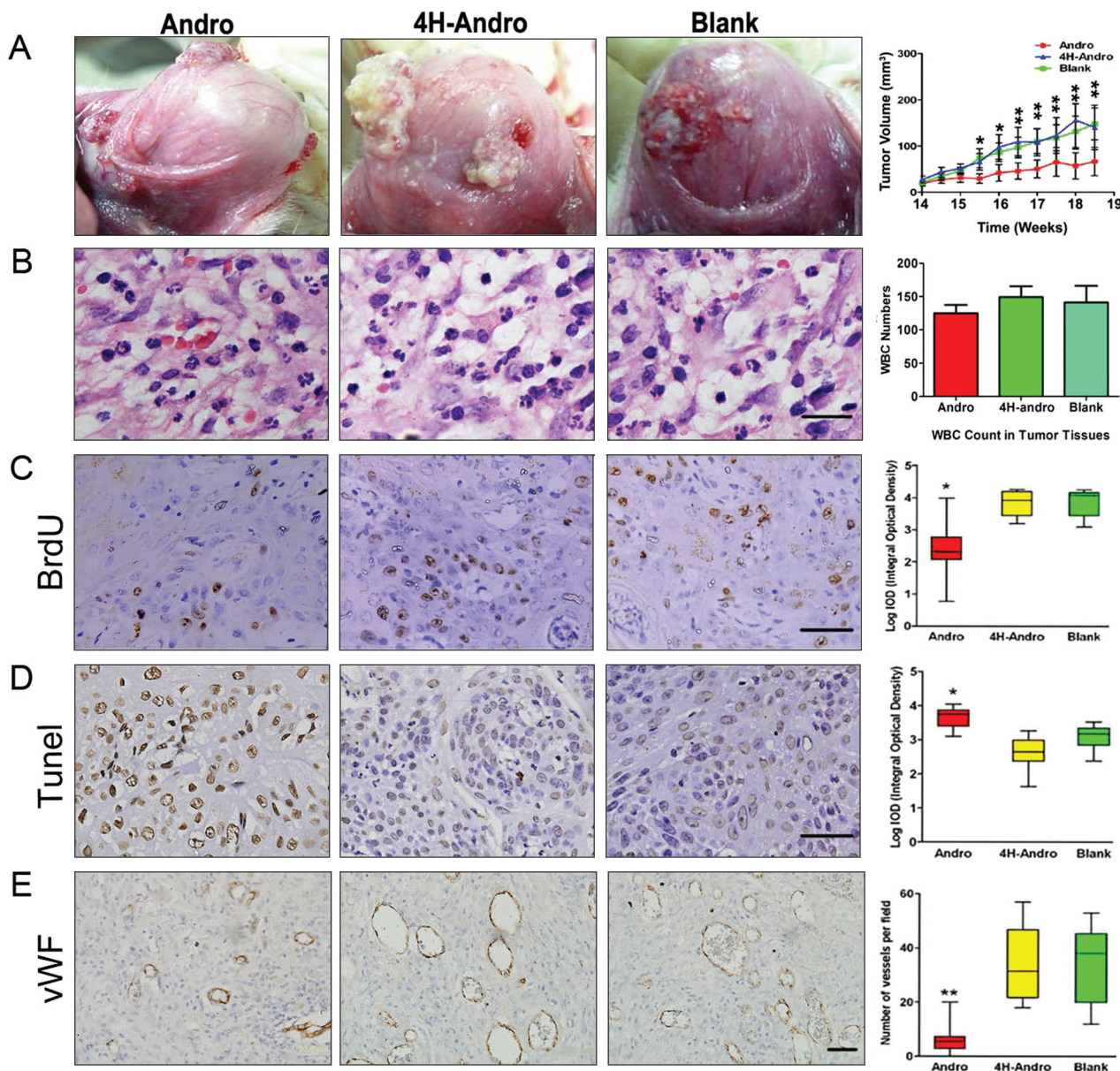


Figure 2. Effects of Andro on chemically induced squamous cell carcinogenesis. Hamsters were treated with saline (n = 14), 4H-Andro (n = 16), or Andro (n = 22). Andro, but not 4H-Andro, reduced tumor growth (A). Leukocyte infiltration in cancerous tissue (B). The immunohistochemical staining of BrdU (C), TUNEL (D), and vWF (E) following saline, 4H-Andro, and Andro treatment. Scale bars, 20 μm for B and 50 μm for C,D,E. *p < 0.05 and **p < 0.01.

Gimenez-Conti, 1993; Li *et al.*, 2002). In the present study, we used this model and demonstrated that DMBA-induced squamous cell carcinogenesis induces NF-κB activation. Importantly, Andro abolished NF-κB activation and potently inhibited tumor growth, attesting to the causal roles of aberrant NF-κB activation during the pathogenesis of chemically induced oral squamous cell carcinoma. Our results indicate a potential clinical correlation of our experimental findings obtained in the chemically

induced squamous cell carcinogenesis in the buccal pouch of hamsters. Consistently, NF-κB activation is known to be closely associated with tumorigenesis and metastasis (Loercher *et al.*, 2004; Zhang *et al.*, 2005; Allen *et al.*, 2007) and sensitivity to chemotherapy and radiotherapy (Wang *et al.*, 1999; Kato *et al.*, 2000) and papilloma virus infection (Mishra *et al.*, 2006), and mastication of tobacco (Sawhney *et al.*, 2007) triggers NF-κB activation in human oral squamous cell carcinoma.

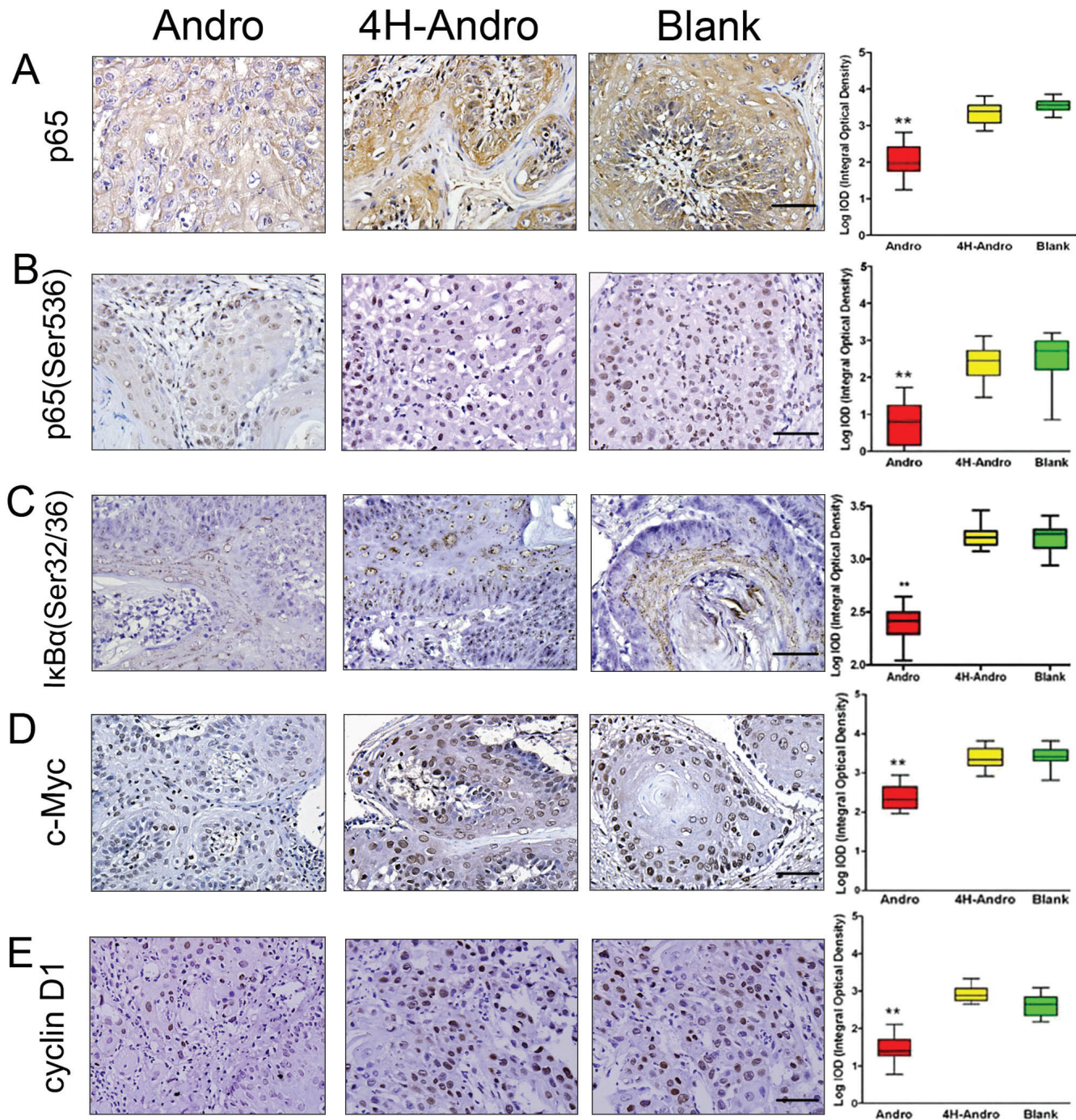


Figure 3. Effects of Andro on NF- κ B activation, proliferation, apoptosis, and angiogenesis. The immunohistochemical staining of total p65 (A), phosphorylated p65 (Ser536) (B), and I κ B α (Ser32/36) (C) or c-Myc (D) and cyclin D1 (E) following saline, 4H-Andro, and Andro treatment. The statistical analysis was performed for least 5 tissue samples from more than 3 hamsters for each group, with ImageTool software. Scale bars, 50 μ m. * p < 0.05 and ** p < 0.01.

The aberrant activation of NF- κ B transcription factors is known to regulate diverse pathological responses of carcinogenesis, including cell-cycle progression and proliferation, apoptosis, and angiogenesis (Deveraux *et al.*, 1998; Li and Verma, 2002; Hayden and Ghosh, 2008). Consistently, we found that Andro suppressed NF- κ B activation for reduced expression of cyclin D1 and c-Myc and decreased BrdU incorporation, leading to inhibition of cell proliferation *in vivo*. In addition,

Andro increased TUNEL staining of DNA fragmentation for the induction of cell apoptosis *in vivo* through targeting p50 and abrogating NF- κ B activation. Notably, Andro treatment also reduced microvessel density (MVD) in the cancerous tissues *in vivo*, suggesting that Andro inhibits angiogenesis during the pathogenesis of chemically induced oral squamous cell carcinoma. Notably, Andro is widely used in China as an over-the-counter medicine, which apparently has no serious

complications or toxic effects reported in patients thus far. Taken together, the *in vivo* experimental findings that Andro significantly inhibits chemically induced oral squamous cell carcinogenesis suggest that the clinical merit for Andro treatment of squamous cell carcinoma warrants further investigation.

To dissect the molecular mechanisms of how inhibition of aberrant NF-κB activation translates into attenuation of chemically induced oral squamous cell carcinoma, we conducted *in vitro* experiments using the human tongue squamous cell carcinoma Tb cells. Consistent with a previous report (Yao *et al.*, 2006) and our *in vivo* findings, treatment of Tb cells with Andro significantly decreased the phosphorylation of p65 (S536) and the expression of c-Myc and cyclin D1. Interestingly, Andro inhibited cell growth and induced cell apoptosis by arresting these squamous carcinoma cells at the G2/M stages *in vitro*. Importantly, our findings that Andro inhibits NF-κB activation for reduced p65 expression and phosphorylation and mitigated κBα phosphorylation are fully consistent with those of previous reports (Jin *et al.*, 2011; Kim *et al.*, 2011). Taken together, our results thus confirm that Andro inhibits tumor growth *in vivo* by the reduction of tumor cell proliferation, induction of tumor cell apoptosis, and suppression of tumor-induced angiogenesis through attenuation of aberrant NF-κB activation.

In summary, analysis of our experimental data suggests that NF-κB signaling plays a critical role during carcinogenesis of chemically induced hamster buccal pouch squamous cell carcinoma. Importantly, by targeting p50 and suppressing NF-κB activation, Andro potently attenuates chemically induced tumor growth for treating oral squamous cell carcinoma.

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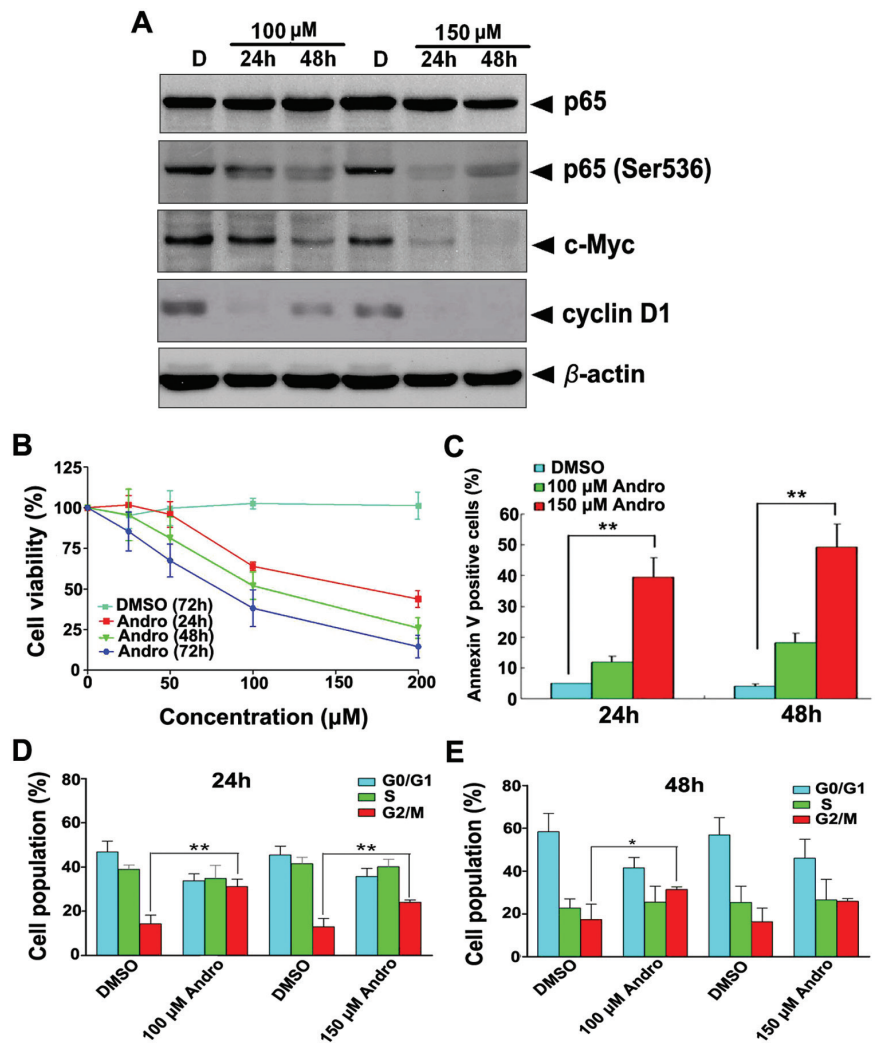


Figure 4. Effects of Andro on NF-κB activation and survival of Tb cells. Time and dose courses of Andro on the expression of total p65, phosphorylated p65 (Ser536), c-Myc, and cyclin D1 (A) and growth (B), apoptosis (C), and G2/M arrest (D,E). The viability of cells was measured with the MTT assay. Results are the mean (± SD) values of 3 separate experiments. *p < 0.05 and **p < 0.01.

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