

Conflict of interest

The authors declare no competing financial interests.

Acknowledgement

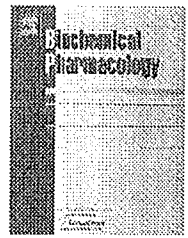
This work was supported in part by priority areas from the Ministry of Education, Culture, Sports, Science and Technology (to F.O., Y.F.-K., and S.K.).

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Review

AhR protein trafficking and function in the skin

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ARTICLE INFO

Article history:

Received 8 July 2008

Accepted 3 October 2008

Keywords:

Subcellular localization

Cell–cell contact

Epithelial–mesenchymal transitions

Wound healing

Carcinogenesis

ABSTRACT

Because aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor, its nuclear translocation in response to ligands may be directly linked to transcriptional activation of target genes. We have investigated the biological significance of AhR from the perspective of its subcellular localization and revealed that AhR possesses a functional nuclear localization signal (NLS) as well as a nuclear export signal (NES) which controls the distribution of AhR between the cytoplasm and nucleus. The intracellular localization of AhR is regulated by phosphorylation of amino acid residues in the vicinity of the NLS and NES. In cell culture systems, cell density affects not only its intracellular distribution of AhR, but also its transactivation activity of the target genes such as transcriptional repressor Slug, which is important for the induction of epithelial–mesenchymal transitions. These effects of AhR observed in cultured cells are proposed to be reflected on the *in vivo* response such as morphogenesis and tumor formation.

This review summarizes recent work on the control mechanism of AhR localization and progress in understanding the physiological role of AhR in the skin. We propose that AhR is involved in normal skin formation during fetal development as well as in pathological states such as epidermal wound healing and skin carcinogenesis.

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doi:10.1016/j.bcp.2008.10.003

1. Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor with basic-helix-loop-helix (bHLH)/PER-ARNT-SIM homology region (PAS) family and is constitutively expressed in various mammalian tissues including lung, liver, thymus, and kidney [1]. AhR is involved in skin carcinogenesis by benzo[a]pyrene [2], teratogenesis in cleft palate [3], and hepatotoxicity [4]. Moreover, recent studies suggest that AhR plays a role in physiological function including immunity [5,6] and reproduction [7,8].

When environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3-methylcholanthrene bind to AhR, the ligand-activated AhR translocates to the nucleus where it binds to its heterodimerization partner AhR nuclear translocator (ARNT) [9]. The heterodimer AhR/ARNT binds to xenobiotic responsive elements, which are enhancer DNA elements located in the 5'-flanking region of the target genes [10]. While AhR activation by exogenous ligands is well investigated, very little is known about the physiological activation of AhR. Many studies using suspension cultures of various cell lines exist to show that AhR-mediated gene expression can be activated in the absence of exogenous ligands [11–13]. When adherent cells are suspended, intracellular signaling may be triggered by the loss of cell–cell contact or cell adhesion. This activation mechanism provides a model to investigate how AhR is activated under the normal physiological conditions.

Exposure to polycyclic aromatic hydrocarbons or topical application of these chemicals elicits inflammatory skin disease [14] as well as tumor formation [15]. This observation suggests that skin may provide clues to elucidate the biological function of AhR. The skin is a dynamic, regenerating organ. When skin is injured, various types of cells including leukocytes, fibroblasts and keratinocytes engage in tissue remodeling [16]. We attempted to study the role of AhR in the wound healing process. In benzo[a]pyrene skin carcinogenesis, it has been reported that AhR^{-/-} mice do not develop tumors [2]. Since stem cells are considered to be the targets for carcinogens, it is very interesting to consider how AhR functions in stem cells. We are also going to discuss the possible roles of AhR on skin carcinogenesis.

2. AhR is a nucleo-cytoplasmic shuttling protein

AhR is a ligand-activated transcription factor and regulates biological responses to a variety of environmental contaminants. When exogenous ligands such as TCDD, benzo[a]pyrene, and 3-methylcholanthrene bind to AhR in cytoplasm, AhR translocates from the cytosol to the nucleus. It is important to investigate the localization of a transcription factor since change in its location is considered to impact gene regulation. Nuclear localization of a lot of nuclear proteins is determined by the nuclear localization signal (NLS) which is used for transport of these proteins to the nucleus through the nuclear pore complex [17,18]. This signal consists of a few short sequences of positively charged amino acid residues, whereas the nuclear export signal (NES) is a short leucine-rich

sequence. We identified both the NLS 13–39 amino acid residues and NES 55–75 amino acid residues in the N-terminal region of AhR [19]. AhR shuttles between the cytoplasm and nucleus using these short peptide signals [20,21]. Subcellular localization of the shuttling protein is determined by the balance between nuclear import and nuclear export. It is reported that the localization is regulated by phosphorylation and dephosphorylation especially of amino acids close to the NLS or NES [22,23]. We found that the ligand-dependent nuclear import of AhR is inhibited by the substitution of aspartic acid for serine-12 or Ser-36, which mimics the negative charge conferred by phosphorylation [24]. It is likely that nuclear import of AhR is regulated by phosphorylation of NLS.

Distribution of AhR in a cell is controlled by its binding protein (Fig. 1). The unliganded AhR exists in cytosolic component as a complex [25], composed of AhR, a dimer of hsp90, p23, and the immunophilin homolog XAP2 [26,27]. XAP2 overexpression in cells is shown to enhance cytoplasmic AhR levels, suggesting that XAP2 is able to stabilize and enhance cellular levels of AhR [28,29]. Petruilis et al. [30] studied the mechanism of cytoplasmic retention of the AhR in the presence of XAP2. They showed that XAP2 hinders the binding of importin β to the AhR complex and proposed that XAP2 alters the conformation of the NLS. In addition, Berg and Pongratz [31] identified the other mechanism of XAP2-induced cytoplasmic localization of AhR. In particular, they showed that XAP2 anchors unliganded AhR to actin filaments since an actin inhibitor, cytochalasin B, blocked this effect.

Several reports demonstrate that the AhR is rapidly degraded via the proteasome pathway following exposure to ligands [32,33]. Davarinos and Pollenz [34] evaluated the function of the NES in the context of AhR degradation. They showed TCDD-induced degradation of the AhR was completely inhibited in the HepG2 cells pretreated with leptomycin B, an inhibitor of nuclear transport mediated by CRM1 (chromosomal maintenance factor 1) [35]. Furthermore, expressed AhR Δ NES protein was degraded to a lesser extent than wild-type AhR [34]. These data suggest that ligand-dependent degradation of AhR is mediated by nuclear export of AhR.

3. Cell density affects AhR localization and activity

We have shown that AhR is a nuclear-cytoplasmic shuttling protein. How are these transport mechanisms regulated under the physiological conditions? We investigated stimuli that affect AhR localization using the human keratinocyte cell line HaCaT in the absence of exogenous ligand [36]. Because growth and differentiation of the cultured keratinocytes is regulated in part by cell density [37,38], effects of cell density on AhR localization were examined. When cells were sparsely inoculated, AhR was predominantly localized in the nucleus. When the cells were subconfluent, AhR was distributed equally both in cytoplasm and nucleus. However, when cells were fully confluent, immunostained AhR was localized predominantly in the cytoplasm. It is suggested that nuclear translocation of AhR is negatively regulated by phosphorylation of serine residues located in its NLS [24]. It is possible that

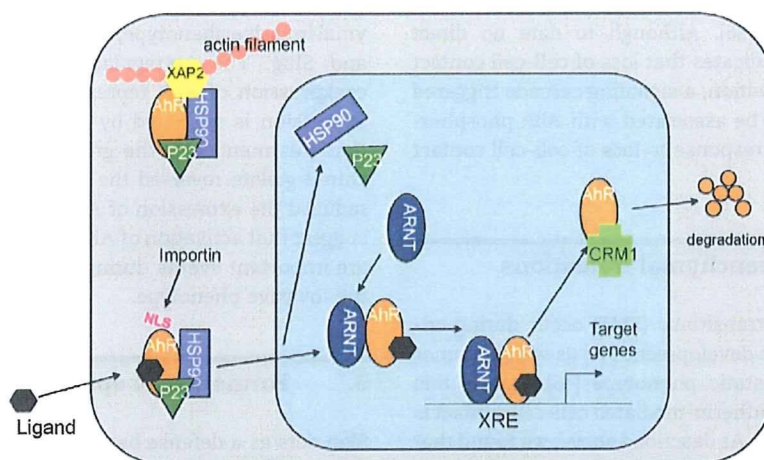


Fig. 1 – Regulation of AhR localization. In cytosol, AhR is complexed with hsp90, p23, and XAP2 (the last of which anchors the ligand-free receptor to the cytoskeleton). Ligand binding results in AhR nuclear transport mediated by importins and is followed by dimerization with aryl hydrocarbon nuclear translocator (ARNT). The AhR/ARNT complex binds to xenobiotic responsive element (XRE), and induces transcription of target genes. AhR protein is exported by chromosome region maintenance 1 (CRM1), followed by degradation in cytosol.

these serine residues are phosphorylated to be anchored in cytoplasm under confluent culture. Using immunoblotting analysis, it was also shown that the relative amount of AhR in the nucleus was gradually decreased in proportion to the cell density. These observations led us to examine whether altered intracellular localization of AhR reflects AhR/ARNT-mediated transcription. Reporter analysis using luciferase cDNA connected to the XRE sequence, revealed that AhR activity was also affected by cell density. While the maximal luciferase activity was observed in subconfluent culture, the luciferase activity decreased to the basal level in confluent culture. These observations showed that subcellular localization and transcriptional activity of AhR were regulated by cell density.

Cell density dependent regulation system is reminiscent of contact inhibition. Cell growth is regulated by cell–cell contact in non-transformed cells. When cultured cells are maintained in low density, cells are actively growing. When cells grow to form confluent monolayer, they stop dividing. Critical anti-proliferative signals are mediated by cell–cell contact. It is very interesting to postulate that AhR is involved in regulation of contact inhibition. Recently, Weiss et al. indicated that TCDD treatment in WB-F344 rat liver cells leads to induction of JunD, resulting in up-regulation of cyclinA which triggers a release from contact inhibition via the AhR [39]. While exposure of confluent cells to TCDD-induced further proliferation, subconfluent cells did not respond to TCDD, suggesting that TCDD treatment specifically interferes with the signaling cascade of contact inhibition. It is suggested that this TCDD effect is an AhR-dependent and ARNT-independent reaction since suppression of AhR expression by siRNA abrogates the TCDD effect in sharp contrast with the suppressed expression of ARNT.

Previous reports showed that localization and transcription activity of AhR were altered in several cell lines when cell–cell

contact was disrupted [11–13]. Recently, Cho et al. reported that suspension culture of C3H10T1/2 fibroblasts in methylcellulose-containing semisolid media resulted in activation of AhR-mediated transcription [40]. The AhR antagonist α -naphthoflavone blocked ligand-stimulated AhR activity, but did not affect the suspension-induced activation of AhR-mediated transcription, implying that the mechanism of the latter is different from that of former. They found that the activation of AhR by ligands can be clearly distinguished from the activation of AhR by the loss of cellular contact. We have shown that AhR is activated in the cells at the wounded edge in *in vitro* wound healing analysis using green fluorescent protein as a reporter of transcriptional activation by AhR/ARNT complex [36], suggesting that the loss of cell–cell contact leads to AhR activation. Owens et al. indicated the importance of Src family kinases in the disruption of cadherin-dependent cell–cell contact [41]. Src kinase is known to be associated with AhR complex [42]. Ligand binding to the AhR causes Src kinase to dissociate from the AhR complex and translocate from cytoplasm to the membrane [43], thereby increasing its own kinase activity which may be required for promoting destabilization of cell–cell contact. They showed that inhibition of the catalytic activity of the Src kinase stabilizes cadherin-dependent cell contacts, suggesting that Src kinase activity is required to disassemble cell–cell contacts. On the other hand, the Src kinase activity stimulates the epidermal growth factor receptor (EGFR) [44] which is known to play an important role in activation of MAPK pathways and other key signal transduction cascades. Activation of MAPK pathways promotes downstream signaling such as ERK and p38. Our results suggested that loss of cell–cell contact generates signals that increase the phosphorylation level of AhR (i.e., phosphorylation of Ser-68 which is located in the NES), thereby causing AhR to accumulate in the nucleus owing to inhibition of the

export activity. It is likely that activated p38 MAPK is involved in this phosphorylation [36]. Although to date no direct experimental evidence indicates that loss of cell–cell contact is the signal for AhR activation, a signaling cascade triggered by Src kinase appears to be associated with AhR phosphorylation and activation in response to loss of cell–cell contact (Fig. 2).

4. Epithelial–mesenchymal transitions

Epithelial–mesenchymal transitions (EMT) occur during critical phases of embryonic development [45] as well as tumor progression to the metastatic phenotype [46]. During this process, disruption of E-cadherin-mediated cell–cell contact is considered to be a key step. As described above, we found that the AhR activation is associated with disruption of cell contact in keratinocyte. This observation prompted us to examine whether AhR is involved in the regulation of EMT. In many types of cancer, the loss of E-cadherin expression is due to transcriptional repression [47,48]. Transcription factors including a family of zinc finger proteins of the Slug/Snail family are implicated in such repression [49–51]. We have shown that AhR participates directly as a transcription factor in the induction of Slug expression in the context of loss of cell–cell contact, which, in turn, regulates EMT [52]. The induced Slug was associated with reduced level of the epithelial marker, cytokeratin 18 and with increased level of the mesenchymal marker, fibronectin. Belguise et al. [53] investigated the control of EMT in breast cancer. Ectopic coexpression of CK2 and NF κ B c-Rel in untransformed

mammary epithelial cells was sufficient to induce a mesenchymal invasive phenotype, in association with induction of AhR and Slug. The up-regulation of Slug was abrogated by coexpression of AhR repressor (AhRR), indicating that Slug expression is regulated by AhR. Furthermore, they showed that treatment with the green tea polyphenol epigallocatechin-3 gallate reversed the malignant phenotype as well as reduced the expression of AhR and Slug. Thus, these results suggest that activation of AhR signaling and induction of Slug are important events during the process of progression into the invasive phenotype.

5. Formation of epidermal tissue

Skin acts as a defense barrier against environmental stimuli and is composed of two layers, the dermis and epidermis. The epidermis is divided into several layers and extends from the basement membrane to the outer surface. The basal layer contains the basal keratinocytes where mitosis occurs. As the keratinocytes mature, they form the spinous layer, granular layer, and stratum corneum which has the barrier function (Fig. 3A). Keratinocytes in the spinous and the granular layer produce differentiation-specific proteins including filaggrin and loricrin which are cross-linked by transglutaminase into cornified envelopes [54].

Fernandez-Salguero et al. [55] investigated skin lesions in AhR-null mice and found an association of structurally abnormal hair fibers, rupture of hair follicles and mixed inflammatory cells infiltrate that progressed to acute ulcers. It has been reported that TCDD affects differentiation of

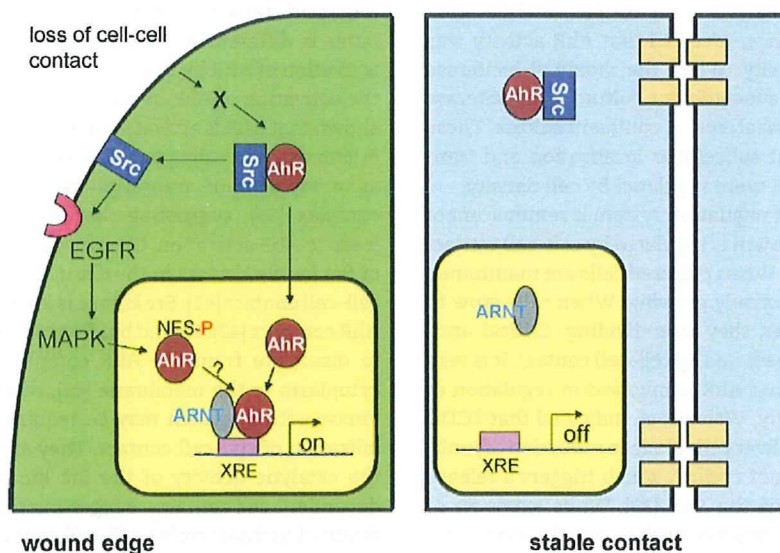


Fig. 2 – A model for AhR localization controlled by cell–cell contact in the *in vitro* wound healing assay. Stable contact (right) mediated by adhesion molecules such as E-cadherin is mechanically disrupted to form wound edge (left). The unknown signal (X) triggered by the loss of cell–cell contact activates AhR, resulting in dissociation of Src from the AhR complex and translocation from the cytosol to the membrane, where its kinase activity required for disruption of cell–cell contact is activated. In addition, Src kinase activity may act as a trigger for the signals such as epidermal growth factor receptor (EGFR)-dependent pathway that induces key signal transduction cascades including MAPK. It is likely that activated p38 MAPK is involved in the phosphorylation of the nuclear export signal (NES) of AhR, which in turn inhibits nuclear export resulting in nuclear accumulation. XRE-mediated transcription is up-regulated by AhR/ARNT complex.

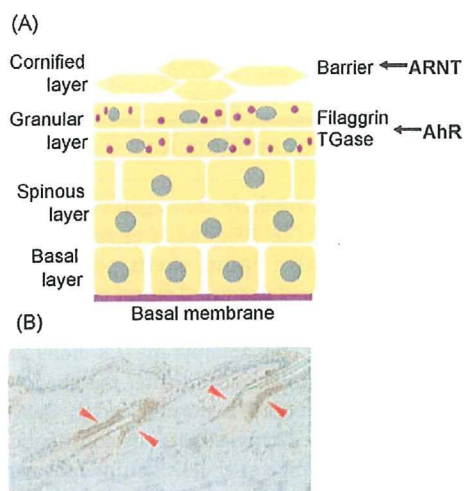


Fig. 3 – (A) Structure of the epidermis, and the possible role of AhR and ARNT. AhR stimulates the expression of filaggrin and transglutaminase 1 (TGase) in granular cells. Barrier formation requires expression of ARNT. (B) Immunohistochemistry for AhR localization on the back skin from 3-wk-old WT C57Bl mice. After excision, the tissue was embedded in O.C.T. compound (Miles, Elkhart, IN) and immediately frozen using liquid carbon dioxide. Frozen tissue was sectioned at 8 μm intervals, and the sections were fixed by 4% formaldehyde before overnight incubation with anti-AhR antibody (BIOMOL, Plymouth Meeting, PA) at 4 $^{\circ}\text{C}$. Arrowheads indicate the site of immunoreactivity including upper part of hair follicle.

keratinocytes. Loetscher et al. [56] examined the effects of TCDD on developing skin of the C57Bl/6J mouse strain. Examination of mouse fetal skin at embryonic day (E) 16 revealed that expression of filaggrin is accelerated in individuals exposed *in utero* to TCDD at E13. They reported that the two putative XREs are present upstream of the transcription start site of human profilaggrin gene. In addition, Du et al. [57] studied the effect of TCDD on differentiation program of human epidermal keratinocytes and showed the induction of transglutaminase 1 at the mRNA and protein levels. This was further confirmed by the increasing transglutaminase 1-mediated cross-linking activity *in situ*. Since there is no XRE motif in the human transglutaminase 1 promoter region, it is unclear how TCDD regulates transglutaminase 1 expression. These investigations suggest that AhR has roles in the modulation of differentiation of keratinocytes.

The function of the skin barrier is partly dependent on terminal differentiation of keratinocytes. The cells differentiate as they move to the skin surface. The stratum corneum is composed of not only insoluble protein such as involucrine and filaggrin but also lipid complex containing cholesterol, ceramides, and fatty acids. Takagi et al. [58] and Geng et al. [59] reported defects in the barrier function in ARNT-deficient mice. They showed that defects in lipid metabolism resulted in failure of the epidermal barrier function. ARNT-disrupted newborn mice died neonatally of severe dehydration caused by water loss. We are interested to investigate whether AhR is

involved in these processes as a heterodimer-partner of ARNT although AhR-null mice have not yet been reported to show such a severe skin phenotype.

To elucidate the AhR function in skin, we examined AhR localization in murine skin by immunohistochemistry. One of the regions in which AhR was detected is the upper part of the hair follicle including the infundibulum (Fig. 3B). Exposure of mammals to TCDD produces an array of pathological manifestations including teratogenesis [3], hepatotoxicity [4], and dermatopathology [60]. Chloracne, a hyperkeratotic skin disorder, is a specific type of acne-like skin disease affecting the hair follicle and inter follicular epidermis and has been used as a hallmark of TCDD exposure in humans. Histopathological analysis of the skin with chloracne has revealed acne-like appearance with hyperkeratosis. In severe cases of acne, the rupture of the infundibulum is associated with inflammation. It is reported that transgenic mice expressing a constitutively active form of AhR in keratinocytes develop severe skin lesions accompanied by inflammation resembling typical atopic dermatitis with increased expression of inflammation-related genes (such as the genes for interleukins and chemokines) [61]. When infundibula maintained in culture were stimulated with interleukin-1 α , hyperkeratosis that was similar to that seen in acne was observed [62]. Taken together, these results prompted us to consider that in response to TCDD exposure, AhR expressed in infundibula is aberrantly activated to induce hyperplasia and inflammation, resulting in chloracne.

6. AhR functions in skin wound healing

Skin wound healing is a dynamic three-phase process: inflammation, tissue formation, and tissue remodeling [16]. Diverse cell types including leukocytes, keratinocytes and dermal fibroblasts participate in each phase. For example, in the early phase of healing, inflammatory leukocytes are recruited to the wounded site. Neutrophils cleanse the wound area of foreign particles, and macrophages release cytokines and growth factors. Keratinocytes migrate and proliferate to cover the wound area, and the dermal fibroblasts synthesize extracellular matrix for tissue remodeling. It is reported that AhR is expressed in these cells [40,63–65]. Here, we investigated the role of AhR in skin wound healing using either wild-type mice, mice heterozygously or homozygously deficient for the AhR gene.

Our methods of full thickness dorsal skin wounding damaged both the epidermis and the underlying dermis. All mice were 8–10 wk old, anesthetized, and received a single 5-mm-diameter excisional wound on the shaved mid-dorsal skin. Mice were kept separately in cages to prevent fighting. Wound closure was determined and expressed as a percentage of the total surface of the wound (Fig. 4). The wounds closed almost completely at day 12 irrespective of AhR genotype. However, in the early phase of healing, wound area decreased faster in the AhR $^{-/-}$ mice than in the wild-type mice and AhR $^{\pm}$ mice. The time needed for 50% closure in wild-type, AhR $^{\pm}$ and AhR $^{-/-}$ mice was 73.7 \pm 3.0 h (n = 50), 59.0 \pm 3.3 h (n = 32) and 47.6 \pm 3.3 h (n = 39), respectively. The difference in time needed for 50% closure between AhR $^{-/-}$

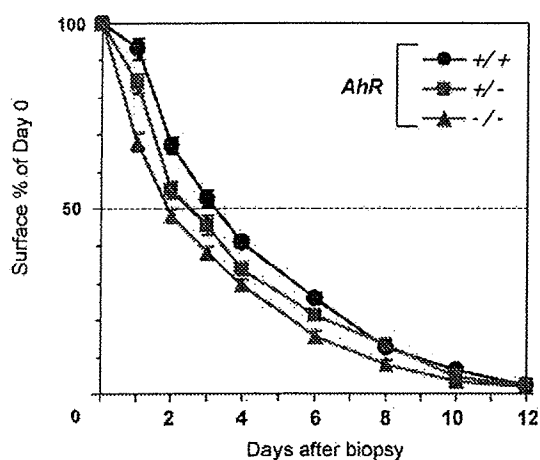


Fig. 4 – Surface areas of the wounds. The back skin of each mouse (8–10 wk) was shaved 1 wk before wounding. A full thickness of mid-dorsal wound (5 mm in diameter) was created and the wound areas were quantified on the indicated days until complete healing, using NIH image software. The surfaces are plotted as percentage of the surface of the wound at day 0 (Mean \pm SE). The surface areas of the wounds at day 0 were not significantly different among genotypes.

and wild-type mice was significant ($P = 1.97E-08$). On the other hand, we did not find any differences in repair processes between wild-type and AhR $^{-/-}$ mice. These data suggest that inactivation of AhR accelerates wound closure during the early phase, which corresponds to the inflammatory phase, of wound healing. These observations are reminiscent of those observed for peroxisome proliferator-activated receptor (PPAR)-mutant mice. Michalik et al. [66] found that wound healing in PPAR $\alpha^{-/-}$ mice was delayed during the first 4 days after injury, suggesting the involvement of inflammation. They further assessed inflammatory infiltration by counting neutrophils and monocytes/macrophages present in the wound bed. Recruitment of neutrophils and monocytes was impaired in the PPAR $\alpha^{-/-}$ mice at day 1. AhR expression has been detected in monocytes and macrophages [63,64] and might affect the function of these cells. Tauchi et al. [61] reported that transgenic mice expressing the constitutive active form of AhR in keratinocytes develop severe skin lesions accompanied by inflammation. It is likely that the skin of AhR $^{-/-}$ mice has a reduced inflammatory phenotype. Aschcroft et al. [67] investigated the role of Smad3, a mediator of TGF- β signaling, in skin wound healing. They reported that mice lacking Smad3 show accelerated wound healing accompanied by reduced inflammatory response (i.e., reduced local infiltration of monocytes) leading to reduced level of TGF- β in the wound site. One possible explanation for the increased rate of re-epithelialization in mice lacking Smad3 is the increase in keratinocyte proliferation due to abrogation of the growth inhibitory effect of TGF- β . In our experiments, we attribute this phenomenon (i.e., faster decrease in wound area in AhR-deficient mice in the early phase of wound healing) to

reduced inflammation. In wild-type mice, AhR may play a supportive role in the inflammatory response.

7. AhR in skin carcinogenesis

AhR is considered to mediate teratogenic and carcinogenic effects. Benzo[a]pyrene (B[a]P), one of environmental chemicals binding to AhR as a ligand, exerts potent carcinogenic activity in several animal species. Topical application of B[a]P produces skin tumors. It has been revealed that the ultimate metabolite of B[a]P (i.e., benzo[a]pyrene-7,8-diol-9,10-epoxide [BPDE], which is synthesized in the metabolic pathway involving cytochrome P450 isoforms) forms DNA adducts and acts as a mutagen. To investigate the contribution of AhR to carcinogenesis, Shimizu et al. [2] examined the response of AhR-deficient mice to B[a]P and found that no tumors appeared in the AhR-deficient mice. They provided direct evidence that AhR is required for skin tumor induction by benzo[a]pyrene.

An important problem in skin cancer research is the identification of the target cells for chemical carcinogenesis. Evidence is accumulating that a subpopulation known as stem cells are the targets of carcinogenesis [68–70]. A number of investigations revealed that initiated cells persist in the epidermis essentially for the life of the animal, suggesting that the initiated cells are not simply proliferating cells but also stem cells. Morris et al. [15] examined the origin of skin tumors. They completely removed the interfollicular epidermis of carcinogen-initiated mice using an abrasion technique although the hair follicles remained undisturbed. The interfollicular epidermis after abrasion regenerated from cells in the hair follicles. Subsequently, tumor promotion was progressing. Although mice with abraded skin developed papillomas and carcinomas, the number of papillomas was half that of mice with unabraded skin. These results suggest that target of tumor initiation is the cells in hair follicles and, to a lesser degree, in the interfollicular epidermis.

One of the cell surface markers of hair follicle stem cells is CD34. Trempus et al. [68] examined whether CD34 participates in two-stage skin carcinogenesis in CD34 knockout (KO) mice since hair follicle stem cells are thought to be a major target of carcinogens. CD34KO mice failed to develop papillomas, suggesting the requirement of stem cells for skin tumor development. Hair follicle stem cells may be a target for carcinogens. If so, stem cell may express AhR to induce P450 isoforms that metabolically activate carcinogens. It has been shown that hematopoietic stem cells, which express functional AhR, have been shown to be a target of polycyclic aromatic hydrocarbons [71]. Treatment of these cells with B[a]P resulted in impairment of cell expansion and inhibition of cell differentiation into various cell lineages including erythrocyte, granulocyte, macrophage, and megakaryocyte. These toxic effects are related to P450-dependent B[a]P metabolite formation. These results suggest that AhR is functional in the system in stem cells.

Thus, it is implied that stem cells are critical targets of carcinogen metabolites produced by P450 isoforms. These metabolites may be involved in the initiation of the stem cell in

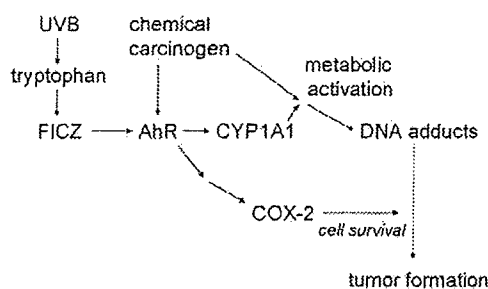


Fig. 5 – Roles of AhR in skin carcinogenesis. CYP1A1 is induced by chemical carcinogens as well as photoproducts of tryptophan [72,73] such as 6-formylindolo[3,2-b]carbazole (FICZ) generated intracellularly by UVB exposure. DNA adducts are formed by reaction with metabolically activated carcinogens. FICZ also induces COX-2 expression through EGFR activation. The COX-2 pathways promote cell survival, resulting in the tumor formation. AhR directly induces CYP1A1 which stimulates generation of initiated cells, and indirectly induces expression of COX-2 leading to tumor promotion.

skin carcinogenesis resulting in the proliferation of the initiated stem cells.

Another risk for skin carcinogenesis is ultraviolet radiation. Photoproducts of tryptophan are known to have high affinity for AhR and are postulated as endogenous ligands [72,73]. The UVB portion (280–315 nm) of the spectrum is a principal risk factor for skin cancer. Fritsche et al. [44] found intracellular formation of the AhR ligand 6-formylindolo[3,2-b]carbazole (FICZ) after UVB irradiation of murine skin and human keratinocyte cell line. AhR activation induces CYP1A1 gene expression and EGFR internalization and subsequent induction of cyclooxygenase-2 (COX-2) gene expression. COX-1 and COX-2 catalyze the first reaction in the conversion of arachidonic acid to prostaglandins. Prostaglandin E₂ is the major product found in UV-exposed skin. In most tissues, COX-1 is constitutively expressed, whereas COX-2 is highly inducible by a variety of inflammatory and tumor-promoting stimuli [74] and is constitutively up-regulated in skin carcinomas [75]. To understand the contribution of COX-1 and COX-2 to UV-carcinogenesis, Fischer et al. [76] performed UV-induced-carcinogenesis experiments using wild-type mice and mice heterogeneously for the COX-1 or the COX-2 gene. While the tumor generations of COX-1± mice and COX-1+/+ mice were essentially similar, the tumor multiplicity in COX-2± mice was reduced to 50–65% and the tumor size was markedly decreased compared with that of wild-type mice. Studies have revealed that UV-induced COX-2 expression contributes to the acquisition of resistance to epidermal apoptosis [77].

In conclusion, UVB exposure induces CYP1A1 and COX-2 in keratinocytes. In this intracellular environment, active carcinogens produced by CYP1A1 may form DNA adducts resulting in generation of initiated cells. Furthermore, as a result of acquiring resistance to apoptosis by induction of COX-2, the initiated cells may be able to clonally expand into detectable skin tumors (Fig. 5).

8. Perspective

Current investigations have revealed that AhR is an important regulator in various tissues even in the absence of exogenous ligands. While identification of endogenous ligands of AhR is one of the most intriguing goals of future study, uncovering the signaling pathway leading to AhR activation in the context of cell–cell contact is also needed. Investigating target genes of AhR/ARNT is also important for elucidation of AhR function. In wound healing, we are interested in the genes that act downstream of AhR in the signaling pathway and are involved in inflammatory agent production such as the release of cytokines. In skin carcinogenesis by benzo[a]pyrene, AhR is believed to be necessary for the induction of CYP1A1 which yields active carcinogens. However, the experimental data indicating that AhR-deficient mice produce no tumors do not exclude the possibility that AhR is involved in cancer progression. It would also be interesting to determine whether induction of epithelial–mesenchymal transitions mediated by AhR is functional in cancer.

Acknowledgements

This study was supported by the Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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An experimental design for judging synergism on consideration to endocrine disruptor animal experiments

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SUMMARY

This paper investigates an appropriate statistical design for an animal experiment to evaluate synergism of two test chemicals. It assumes a certain number of animals are divided into groups, each of which is treated with a combination of dose levels of two chemicals. A design is identified by the set of group size for each combination of doses, including the case where the dose of either one chemical is zero. The power of *t*-test to detect synergism by positive surplus of response on a simultaneous administration group from the additivity plane composed of the responses on single administration groups is adopted as the criterion for the appropriate design. The applicable design is investigated for the application to real cases of endocrine disruptor study conducted at the National Institute of Health Sciences of Japan.

It revealed that the dose level of the simultaneous administration group should be located inside or on the boundary of a triangular region and that the total number of animals should be the same as those for single administration groups. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS: additivity; animal experiment; experimental design; endocrine disruptor; synergism; triangular region

1. INTRODUCTION

In the past, environmental pollutants were regulated according to individual effects. However, recently, there has arisen the problems of combinations of complex pollutants, and regulations that address synergism have become necessary. As a result, experimental researches have been conducted on pollutant synergism. The investigation by Kanno, one of the authors (Kanno *et al.*, 2001) on the synergism of endocrine disruptors, using the rodent uterotrophic assay, is an example of such researches.

In our experiments using multiple test substances, dividing animals such as rats into multiple groups of single administration and simultaneous administration, we estimated the response when there is no synergism based on the response in the single administration group to investigate whether the response in the simultaneous administration group exceeds the estimated response.

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For the data analysis method used in this type of animal experiment to investigate synergism, Kelly and Rice (1990) proposed a method to evaluate the dose–response curve by smoothing method. Gennings and Carter (1995) and Gennings *et al.* (1997), on the other hand, proposed a method to evaluate synergism by using a model in which the response becomes flattened when there is no synergism. Using a similar plan to that of Gennings *et al.* (1997), Matsunaga *et al.* (2003) proposed a method to evaluate the difference in the responses with simultaneous administration of two substances from those estimated by applying an additive model to the data of single administration of each substance. They applied their proposed method to the actual data analysis. Other data analysis methods are also cited in Laska and Meisner (1989) and Machado and Robinson (1994).

For the experimental design evaluating the synergism, Hasegawa *et al.* (1996) proposed the experimental design of animal experiment for five or ten chemical mixture, and Straetemans *et al.* (2005) investigated a fixed-ratio design on *in vitro* study. Abdelbasit and Plackett (1982) and Tan *et al.* (2003) are also related to this issue. However, the situation of these researches differs from our case study. Our interest is limited to simply checking whether the effects of the chemicals are additive or not. And in the animal experiments, some assumptions and limitations are generated for the applicable information and experimental conditions.

In this kind of research, we thought that the problem of the experimental design is to determine appropriate dose levels for simultaneous administration and to select the appropriate number of animals for allocation to the dose levels. However, in the past research, the research directly related to this problem by the animal experiment was not found. Accordingly, based on the analysis methods proposed by Matsunaga *et al.* (2003), we investigated what type of design would be appropriate.

The paper is organized as follows. Section 2 introduces the conditions in the case study that motivated this paper, while Section 3 formulates the issues. Section 4 derived the appropriate design corresponding to the case studies dealt with this paper. Finally, Section 5 provides Conclusions and Discussions for future issues.

2. MOTIVATING CASE

According to the World Health Organization, an endocrine disruptor is defined as “an exogenous substance or mixture that alters function(s) of the endocrine system and causes adverse health effects in an intact organism, or its progeny or (sub)populations.”

The effect of endocrine disruptors is not stimulated directly at the site of the adverse effect, but is mediated by the signal and occurs through nuclear receptor. Furthermore, there is more than one signal transduction system in humans and animals. Because nuclear receptors and transcription factors are redundant, there may be an interaction between different pathways, which leads to possible synergistic endocrine disruption action. Here the definition of “synergistic” is that if two chemicals produce the same endpoint, they bring about a larger response as compared to the anticipated response when these chemicals are purely added. Consequently, it is very important to realize the synergism between two endocrine disruptors such as “Genistein” and “bisphenol A (BPA)” through animal experiments for explaining the mechanism of action.

Both Genistein and BPA bind to estrogen receptors and elicit estrogenic responses to an organism including uterotrophic responses. Genistein is a phytoestrogen found relatively abundant in soybeans and its derivative foods. BPA is the basic monomer of polycarbonate plastic and epoxy resins widely used as a lining for food and beverage cans, in hard plastic baby and water bottles, toys, dental sealants, etc. It has been reported that BPA monomer can leached out to food and drinks especially when the

polymerization process is incomplete and/or the plastic is aged. These two estrogenic compounds can be found very commonly in our food environment. Therefore, it is of great importance from the point of the safety regulations to examine whether the combined effect is additive or synergistic.

For the experimental design of the research based on this background, some assumptions and limitations were generated for the applicable advanced information and experimental conditions. In the interests of simplicity, when explaining synergism research for two substances, the limitations are as follows.

First, before conducting the experiment that investigates synergism, advance knowledge can be obtained to some extent on dose–response curves for the single administration from preliminary experiments using each substance. Therefore, if the dose–response curve is nonlinear, by appropriate transformation of variables for the dose and response, it is possible to assume the dose–response curve to be approximately linear.

Second, the maximum dose of each substance used in the experiment is limited. We, for example, recognize in our experiments that the signal transduction system amplifies the signal at significantly smaller doses as compared to the dose used in normal toxicity studies. Other toxicities appear with higher doses, so that the endocrine disruption effect to be investigated is concealed. Because these maximum doses, $D_{A\max}$ and $D_{B\max}$, generally can be obtained through preliminary experiments, the range of dose levels used in the experiments can be limited.

Third, because various kinds of test substances are to be investigated, the number of animals, n , used in each experiment is relatively small. In our actual experience, the number of animals is approximately 40–50.

Fourth, the fundamental form of the experiment for investigating synergism is roughly decided. In an experiment using two substances A and B , we set single administration groups of G_{00} (at a dose level of 0), G_{A2} , and G_{B2} (at the maximum dose levels of $d_{A2} = D_{A\max}$ and $d_{B2} = D_{B\max}$, respectively), and groups G_{A1} and G_{B1} (at the middle dose levels of d_{A1} and d_{B1} , respectively), and administer the test substances after assigning the same number of animals n_s in every group. Independent of this, we set one or more simultaneous administration groups of G_{AB} with dose levels of the two substances at d_A and d_B . We measure responses by performing the experiment with this type of design, and estimate dose–response curves by forecasting synergism from the single administration group to confirm whether the response obtained in the simultaneous administration group is larger compared to that estimated.

Fifth, the observed response is usually quantitative variable such as the uterine weight of rats, which generally shows normal distribution, because it is difficult to define the additivity/synergism for the response in qualitative values.

Under the above conditions, what should be questioned for the design is what the most appropriate dose for simultaneous administration is, and whether to have more or less animals for the simultaneous administration group as compared to the single administration group. In order to obtain guidelines for these, this paper generalizes and formulates the above mentioned problems to make numerical evaluations under some conditions.

3. FORMULATION OF PROBLEM

3.1. Definition of synergism

In order to simplify the discussion, hereafter, we assume that there are two test substances denoted by A and B .

There have been many discussions in the past for how to define the terms additivity, synergism, and antagonism. Synergism is not defined unconditionally (Hewlett and Plackett, 1959; Berenberm, 1989).

Table 1. Difference between factorial design and triangular design

Dose of <i>B</i>	Dose of <i>A</i>		
	d_{A0}	d_{A1}	d_{A2}
(a) Factorial design			
d_{B0}	(1)	(2)	(3)
d_{B1}	(4)	(6)	(7)
d_{B2}	(5)	(8)	(9)
(b) Triangular design			
d_{B0}	(1)	(2)	(3)
d_{B1}	(4)	(6)	
d_{B2}	(5)		

Single chemical is administered at (1)–(5), whereas combination of two chemicals is administered at (6)–(9).

In fact, in the general remarks of these studies, many ideas are introduced for discussing synergism such as “independent joint action,” “potentiation,” “simple similar action,” “complex action,” and “dissimilar action.” We will first explain the definition of synergism that is adopted in this paper.

From the standard statistical viewpoint, the dosages set certain dose level for the respective two substances as shown in Table 1(a). If the response at the simultaneous dose level is the sum of the effects generated by single substances, then the effect is considered to be additive. On the other hand, if it is large, there is a positive interaction and the effect is synergistic.

However, with toxic responses like endocrine disruption action, this point of view is not appropriate. Because, for this toxic response, as pointed out by, for example, Hasegawa *et al.* (1996), it is impossible to establish response linearity at doses that exceed the maximum dose for the respective substances, and thus it is impossible to determine whether a positive interaction is attributable to synergism or nonlinearity. Therefore, the following definition that expresses the tenets of Hewlett and Plackett (1959) by isobologram is adopted in this paper.

Label the expected response at dose d_A and d_B (doses for *A* and *B*) as $f(d_A, d_B)$. Also, label the single administration dose of *A* that results in an arbitrary response E as D_A , so that $f(D_A, 0) = E$. Similarly, label the dose of substance *B* that has expectation E as D_B . In the cases that motivated this study, *A* and *B* generate their responses in a similar stimulation process, the expected effects are proportional to the doses of *A* and *B*, and the effects of the two substances are additive. If these conditions hold, then $f(d_A, d_B) = E$ whenever (d_A, d_B) satisfies Equation (1).

$$\frac{d_A}{D_A} + \frac{d_B}{D_B} = 1 \quad (1)$$

The reason is that because a combination of dose levels like this represents simultaneous administration of *A* and *B* at an arbitrary ratio, using the amount that brings about a response of the same magnitude. If a response of magnitude E is consequently generated as expected, there is no special combined effect between the two chemicals. In this paper, when this relationship holds, the effect of the two substances is additive, or the two substances satisfy additivity.

On the other hand, if the two substances generate a synergistic response in different stimulation processes, it is considered that $f(d_A, d_B) > E$ is established with respect to an arbitrary (d_A, d_B) that satisfies Equation (1). In this paper, when this relationship is established, the two substances are synergistic, or satisfy synergism.

3.2. Terminology, notation, and assumption

The two-dimensional plane by plotting d_A (dose of A) on the x -axis and d_B (dose of B) on the y -axis is referred to as the dose plane, and the three-dimensional space by plotting the response on the z -axis above the dose plane is referred to as the response space.

In the experiment, the response is measured for each individual animal. The response that is measured is called the response variable, and is generally expressed by the symbol Z . The response variable Z measured for each individual is set as a random variable that follows a normal distribution independent of other individuals. Since it is assumed that the endpoint is organ weight as a target for application in the case study, such as endocrine disruptor study, it is considered that the assumption of the normal distribution is empirically valid. When the dose of the two substances administered is (d_A, d_B) , the expected value is $E\{Z\} = f(d_A, d_B)$.

For single administration, that is, when the dose of one test substance is 0, Equation (2) can be assumed concerning the dose–response curve f .

$$f(d_A, 0) = \beta_0 + d_A\beta_A, f(0, d_B) = \beta_0 + d_B\beta_B \quad (2)$$

This assumes the dose–response curve for single administration to be linear. With this assumption, $f(d_A, d_B)$ is expressed by Equation (3). The two substances are additive if the hypothesis H_0 of Equation (4) holds, while synergistic if the hypothesis H_1 holds.

$$f(d_A, d_B) = \beta_0 + d_A\beta_A + d_B\beta_B + \Delta(d_A, d_B) \quad (3)$$

$$H_0 : \Delta(d_A, d_B) = 0, H_1 : \Delta(d_A, d_B) > 0 \text{ for all } (d_A, d_B) \quad (4)$$

The value of the dose used in the experiment is called the dose level, the collection of animals allocated for the dose level is called a group, and the number of animals for each group is called the group size, and the dose level of the group on the dose plane is called the group point. With this terminology, it is defined that “design is the set of group point and group size.”

For numerical evaluation described in the next section, five groups of group size n_s for single administration and one group of group size n_m for simultaneous administration as described in Table 1(b) are assumed as the design. For the single administration group, the group points are set to be $(0, 0)$, $(d_{A1}, 0)$, $(d_{A2}, 0)$, $(0, d_{B1})$, $(0, d_{B2})$, and the response variables are distributed as normal with variance σ_s^2 . For the simultaneous group, the group point is set to be (d_A, d_B) , and the response variable is distributed as normal with variance σ_m^2 .

Let the sample mean of response variable in each group be Z_{00} , Z_{A1} , Z_{A2} , Z_{B1} , Z_{B2} , and Z_{AB} , respectively. It is assumed that Z_{00}, \dots, Z_{B2} are distributed as normal with the mean of Equation (2) and the variance σ_s^2/n_s , while Z_{AB} is distributed as normal with the mean of Equation (3) and the variance σ_m^2/n_m .

3.3. Criterion for the appropriate design

As criterion for the most appropriate design, it is natural to use the power in the hypothesis test of “ H_0 versus H_1 .” Because the model is a linear model and the hypothesis is a linear hypothesis, a one-sided

t -test (or Welch test) with Equation (5) is naturally set as the test statistic.

$$T = \frac{\hat{\Delta}}{\sqrt{\hat{V}(\hat{\Delta})}} \quad (5)$$

Here, $\hat{\Delta}$ is the least square estimator of Δ , and the denominator of the statistics is the square root of the variance estimator.

The critical value of this test statistics is $t(\nu, \alpha)$, the upper 100α percentile of the t -distribution with degree of freedom ν , and the test is a one-sided test. In other words, A and B are judged to be synergistic when $T > t(\nu, \alpha)$.

This test, in short, detects the synergism when there is a statistically significant difference between Z_{AB} and the estimate obtained from the single administration groups assuming the dose-response surface under H_0 .

4. EXAMPLES OF RECOMMENDED DESIGN

4.1. Real examples motivated the problem

We conducted many experiments and selected endocrine disruptor study as case study. This study was performed using triangular design such as Table 1(b). This design consisted of seven dose groups which included five group points for single administration and two group points for simultaneous administration. The endpoint was uterine weight gain and the main purpose was to evaluate whether the combined effect was synergistic or not. In order to explain the characteristic of the data, we took up two real examples. The details of these data are as follows.

Example 1. Chemical A: genistein (mg/kg), chemical B: BPA (mg/kg)

1. Group points for the single administration: $(d_A, d_B) = (0, 0), (12.5, 0), (25, 0), (0, 35), (0, 70)$.
2. Group points for simultaneous administration: $(d_A, d_B) = (6.25, 17.5), (12.5, 35)$.
3. Group size: $n_s = 6, n_m = 6, n = 42$.
4. Mean \pm standard deviation of observed values

$$(d_A, d_B) = (0, 0) : 84.0 \pm 7.1$$

$$(d_A, d_B) = (12.5, 0) : 111.2 \pm 9.3, \quad (d_A, d_B) = (25, 0) : 149.5 \pm 33.7$$

$$(d_A, d_B) = (0, 35) : 138.4 \pm 16.2, \quad (d_A, d_B) = (0, 70) : 181.0 \pm 24.4$$

$$(d_A, d_B) = (6.25, 17.5) : 141.1 \pm 11.4, \quad (d_A, d_B) = (12.5, 35) : 180.2 \pm 26.8$$

5. Estimated value of Δ : 15.6.
6. Result of t -test: Significant in one-sided Welch test with significance level 2.5% $T = 2.19, \nu = 19, p = 0.02$.

Example 2. Chemical A: diethylstilbestrol ($\mu\text{g}/\text{kg}$), chemical B: genistein (mg/kg)

1. Group points for the single administration: $(d_A, d_B) = (0, 0), (0.1, 0), (0.2, 0), (0, 12.5), (0, 25)$.
2. Group points for simultaneous administration: $(d_A, d_B) = (0.05, 6.25), (0.1, 12.5)$.

3. Group size: $n_s = 6, n_m = 6, n = 42$.
4. Mean \pm standard deviation of observed values

$$(d_A, d_B) = (0, 0) : 91.2 \pm 16.2$$

$$(d_A, d_B) = (0.1, 0) : 92.3 \pm 9.7, \quad (d_A, d_B) = (0.2, 0) : 96.5 \pm 5.4$$

$$(d_A, d_B) = (0, 12.5) : 165.4 \pm 27.0, \quad (d_A, d_B) = (0, 25) : 220.8 \pm 27.5$$

$$(d_A, d_B) = (0.05, 6.25) : 141.8 \pm 12.8, \quad (d_A, d_B) = (0.1, 12.5) : 183.5 \pm 10.3$$

5. Estimated value of Δ : 19.5.
6. Result of t -test: significant in one-sided Welch test with significance level 2.5% $T = 3.98, \nu = 31, p < 0.01$.

From the above results, we suggested that the combinations for these two agents were synergistic. The synergism was observed in the real situations such as endocrine disruptor study.

4.2. Recommended group point selection

The example introduced in the preceding section has two simultaneous administration groups. In this section, we investigate the most appropriate dose level among several dose levels in the case of one simultaneous administration group. The conditions in the investigation are as follows. The conditions are set pursuant to the example in the preceding section except for assuming $\sigma = 1$ without losing any generality.

4.2.1. Fixed condition.

1. Group points for single administration: $(d_A, d_B) = (0, 0), (1, 0), (2, 0), (0, 1), (0, 2)$.
2. Group size: $n_s = 6, n_m = 12$.
3. Parameters in the dose-response curve: $\beta_0 = 1.0, \beta_A = 1.0, \beta_B = 1.0$.
4. Variance σ^2 : $\sigma_s^2 = \sigma_m^2 = 1.0$.
5. Nominal significance level of t -test: one-sided 2.5%.

4.2.2. Varied condition.

6. Group points for simultaneous administration: $d_A = 0.1(0.1)2.0, d_B = 0.1(0.1)2.0$.
7. Strength of synergism Δ :
 - Case 1 (constant case): $\Delta = 1.0$
 - Case 2 (square root case): $\Delta = 0.8\sqrt{(d_A + d_B)}$
 - Case 3 (linear case): $\Delta = 0.6(d_A + d_B)$

Numerical calculations were performed to calculate the power under the above conditions. The left-hand side of Figures 1–3 shows a three-dimensional display on the vertical axis above the dose plane of the power in Cases 1–3, respectively. On the other hand, the right-hand side of Figures 1–3 represents power functions when $d_A = d_B$ at the dose level for simultaneous administration group.

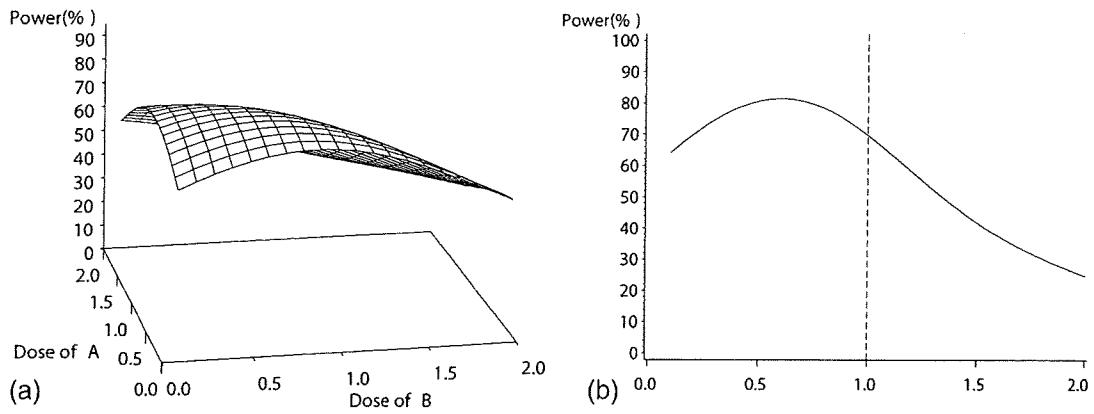


Figure 1. Power surface on the (a) dose plane and power function on the (b) dose for a simultaneous administration group with a constant surplus case (Case 1)

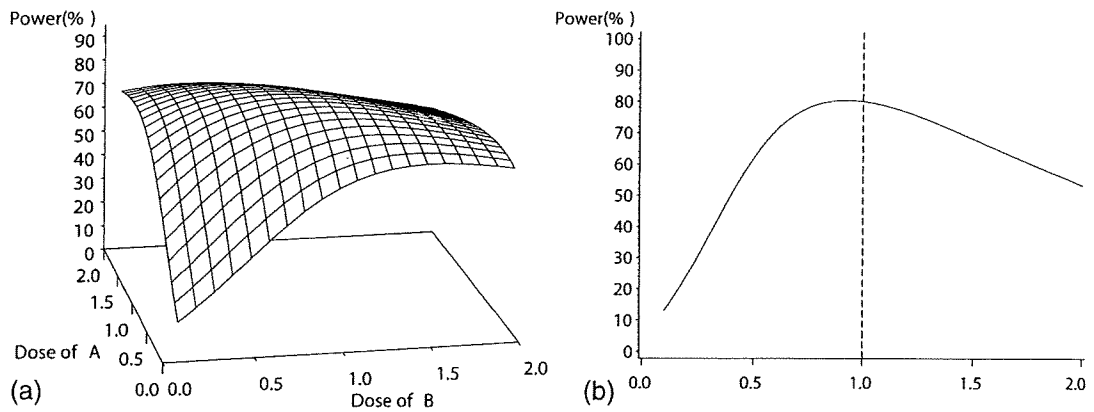


Figure 2. Power surface on the (a) dose plane and power function on the (b) dose for a simultaneous administration group with a square root surplus case (Case 2)

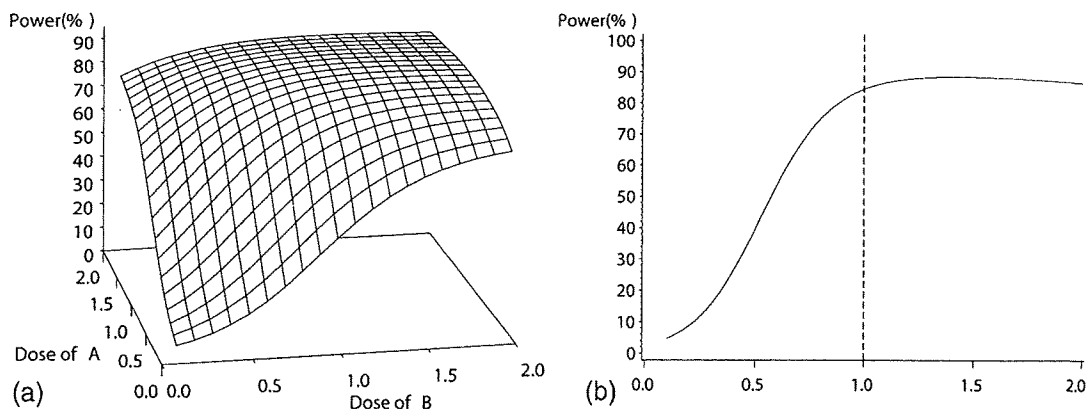


Figure 3. Power surface on the (a) dose plane and power function on the (b) dose for a simultaneous administration group with a linear surplus case (Case 3)