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Original Research Article

Cytotoxic Evaluation of Niclosamide via Saccharin-Based Multicomponent Crystal: Insights from *in Vitro* MTT Assay

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ABSTRACT

Breast cancer remains a major health burden among women globally. While targeted therapies continue to evolve, affordable and effective alternatives are still needed. Niclosamide, an FDA-approved anthelmintic, shows promise for drug repurposing due to its anticancer activity, though its poor solubility and bioavailability limit clinical use. To address this, cocrystallization with saccharin, a GRAS-status coformer, was employed to enhance niclosamide's physicochemical properties without altering its pharmacological effect. This study evaluates the cytotoxic effect of pure niclosamide and the niclosamide–saccharin cocrystal on T47D breast cancer cells, synthesized via the solvent drop grinding (SDG) method and assessed using the MTT assay with microplate spectrophotometric analysis. The results demonstrated that both pure niclosamide and its multicomponent crystal exhibited cytotoxic effects on T47D cells. Notably, the niclosamide–saccharin crystal showed a lower IC_{50} value (0.90 µg/mL) compared to that of pure niclosamide (1.39 µg/mL), indicating enhanced potency. A significant decrease in T47D cell viability was observed, suggesting that the multicomponent crystal formulation may augment the anticancer efficacy of niclosamide. In conclusion, the formation of a niclosamide–saccharin multicomponent crystal significantly enhances its cytotoxic activity against breast cancer cells *in vitro*. These findings underscore the potential of cocrystallization as a strategic formulation approach to improve the therapeutic performance of repurposed drugs in cancer treatment.

***Keywords*:** Niclosamide, Saccharin, Multicomponent crystal, Breast cancer, Drug repurposing, Cytotoxicity.

Introduction

Breast cancer remains one of the leading causes of cancer-related mortality among women worldwide. According to global cancer statistics, the incidence of breast cancer continues to rise, posing significant public health and economic challenges, especially in low- and middle-income countries, including Indonesia.[1] Despite advances in early detection and treatment, many patients still face limited access to effective therapies, making the development of affordable and accessible anticancer strategies a pressing priority.[2,3] One promising direction in anticancer drug discovery is drug repurposing, which involves identifying new therapeutic uses for existing, approved drugs. This approach offers several advantages, including reduced development costs, well-established safety profiles, and accelerated clinical translation.[4,5] Among the repurposed drug candidates, niclosamide, an FDA-approved anthelmintic agent, has shown encouraging antitumor activity across various cancer types, including breast, colon, prostate, and lung cancers.[6,7]

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Niclosamide is known to modulate multiple cancer-related signaling pathways, such as Wnt/*β*-catenin, mTORC1, STAT3, and NF-κB.[8–11] However, the clinical application of niclosamide as an anticancer agent is hindered by its poor aqueous solubility and limited oral bioavailability.[12,13] These physicochemical limitations result in subtherapeutic drug concentrations at the target site, reducing its overall efficacy *in vivo*.[14] Therefore, overcoming these pharmaceutical barriers is essential to unlock the full therapeutic potential of niclosamide in oncology.[15] In recent years, cocrystallization has emerged as a powerful strategy in pharmaceutical development to enhance the solubility, dissolution rate, and stability of poorly soluble drugs without altering their pharmacodynamic properties.[16–19] Multicomponent crystals (cocrystals) are formed through non-covalent interactions between an active pharmaceutical ingredient (API) and a coformer.[20,21] Saccharin, a Generally Recognized As Safe (GRAS) compound, has been widely used as a coformer due to its strong hydrogen-bonding capability, biocompatibility, and regulatory acceptability.[22,23] This study focuses on the preparation and evaluation of a niclosamide–saccharin multicomponent crystal to improve the drug's cytotoxic potency against T47D human breast cancer cells. The crystal was synthesized using the solvent drop grinding (SDG) method, and its cytotoxic activity was assessed using the Microculture Tetrazolium Test (MTT) assay. The findings of this research aim to provide further insight into the potential of cocrystal engineering as a formulation strategy to enhance the anticancer efficacy of repurposed drugs, particularly in the treatment of breast cancer.

Materials and Methods

Materials and Instruments

Niclosamide (≥98% purity) was purchased from Sigma-Aldrich (USA), and saccharin was obtained from Merck (Germany). Ethanol (analytical

grade) was supplied by Brataco Chemical (Indonesia). The T47D human breast cancer cell line was obtained from Prof. Tatsuo Takeya (Nara Institute of Science and Technology, Japan). Reagents for cell culture, including Roswell Park Memorial Institute 1640 (RPMI) (Gibco, New York, USA), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA, were sourced from Gibco (Thermo Fisher Scientific, USA). The MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich (USA). Others include a microplate reader (Bio-Rad, USA) at 550 nm, a CO_2 incubator (Thermo Scientific, USA), a Class II biosafety cabinet (Thermo Scientific, USA) and an inverted microscope (Nikon, Japan).

Synthesis of Niclosamide–Saccharin Multicomponent Crystal

The niclosamide–saccharin multicomponent crystal was synthesized using the solvent drop grinding (SDG) method, which involves co-grinding the drug and coformer in the presence of a few drops of solvent to facilitate cocrystallization[24]. Detailed optimization steps and extended characterization results of the SDG process will be reported separately in our forthcoming manuscript.

MTT Assay for Cell Viability

The MTT assay was employed to quantify cell viability based on mitochondrial reductase activity, using the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. T47D breast cancer cells (1×10^4 cells/well) were plated in 96-well plates with RPMI-1640 medium (supplemented with 10% FBS and 1% penicillin-streptomycin) and allowed to adhere for 24 h (37°C, 5% CO_2). Test compounds (pure niclosamide and niclosamide-saccharin cocrystal) were serially diluted (0.1–100 µg/mL) in 0.1% DMSO/RPMI-1640 and applied to cells for 48 h, with untreated and vehicle (0.1% DMSO)-treated cells serving as controls (n=3 replicates/group). Post-treatment, 10 µL MTT (5 mg/mL PBS) was added per well, followed by 4 h incubation to form formazan crystals. The supernatant was aspirated, and crystals were solubilized with 100 µL DMSO. Absorbance at 550 nm (Bio-Rad microplate reader) was normalized to untreated controls to determine viability percentages. Dose-response curves generated via linear regression yielded IC^{50} values. The experimental procedure was adapted from a previously reported method with slight modifications.[25]

Cell Morphology Observation

T47D breast cancer cells were seeded in 6-well plates at a density of 1×10^4 per well and incubated overnight at 37 °C in a humidified atmosphere containing 5% CO_2 to allow cell attachment. The cells were then treated with pure niclosamide or niclosamide–saccharin cocrystal at various concentrations for 48 hours. After treatment, the morphological characteristics of the cells were examined using an inverted microscope (Nikon, Japan) at 100× magnification. Viable cells were identified by their normal polygonal shape and adherence to the culture plate, whereas cells undergoing cytotoxic effects were characterized by rounding, shrinkage, and detachment from the surface. Representative images were captured for documentation.

Statistical Analysis

All experimental data were expressed as mean ± standard deviation (SD), n=3. Statistical analysis was conducted using GraphPad Prism software (version 8.4.0 GraphPad Software, San Diego, CA, USA). The IC_{50} value was calculated using linear regression analysis of the dose–response data, where cell viability (%) was plotted against the logarithm of the tested compound concentrations, and the concentration corresponding to 50% cell viability was obtained from the regression equation. Statistical significance among groups was assessed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. A p-value of less than 0.05 was considered statistically significant.

Results and Discussion

The cytotoxicity assay using the MTT method demonstrated that both pure niclosamide and the niclosamide–saccharin cocrystal reduced the viability of T47D breast cancer cells in a dose-dependent manner after 48 h of treatment (Table 1, Figure 1). At the highest concentration tested (100 µg/mL), the remaining viable cells were only 1.747 ± 0.081% for pure niclosamide and 0.943 ± 0.081% for the cocrystal, compared with the untreated control. The IC_{50} values were 1.39 µg/mL for pure niclosamide and 0.90 µg/mL for the cocrystal, indicating an enhancement of cytotoxic potency following cocrystallization. The generated dose–response curves showed a sharp decline in cell viability above 1 µg/mL for both formulations, with a steeper slope observed for the cocrystal, reflecting its ability to exert cytotoxic effects at lower concentrations.

Table 1: Cytotoxic Effect of Niclosamide and Niclosamide–Saccharin Cocrystal on T47D Breast Cancer Cells Measured by MTT Assay.

Concentration (µg/mL)	Pure Niclosamide		Niclosamide–Saccharin Cocrystal	
	Mean Absorbance ± SD	Mean % Viability ± SD	Mean Absorbance ± SD	Mean % Viability ± SD
Untreated	0.780±0.022	100 ± 0.000	0.780±0.022	100 ± 0.000
100	0.085±0.001	1.747 ± 0.081****	0.079±0.001	0.943 ± 0.081****
10	0.157±0.003	11.417 ± 0.454****	0.167±0.002	13.300 ± 0.242****
1	0.278±0.001	29.010 ± 0.140****	0.424±0.019	49.620 ± 2.619****
0.1	0.844±0.031	109.057 ± 4.367***	0.624±0.057	77.923 ± 8.008***
IC_{50} (µg/mL)	1.39		0.90	

Data are presented as mean ± standard deviation (SD), n = 3. Cell viability was calculated relative to the untreated control. Absorbance was measured at 550 nm after 48-hour treatment. IC_{50} values were calculated using linear regression. Statistical significance was analyzed using one-way ANOVA followed by Dunnett's ($p < 0.05$). ***$p < 0.001$, ****$p < 0.0001$ compared to Untreated.

This improvement in potency can be attributed to enhanced physicochemical properties resulting from the cocrystallization process. Niclosamide belongs to the Biopharmaceutics Classification System (BCS) Class II, characterized by low solubility and high permeability, where solubility is the rate-limiting step for bioavailability.[26] Previous studies have reported that forming cocrystals with coformers significantly increases the solubility and dissolution rate of niclosamide,[26] thereby improving the availability of drug molecules to interact with intracellular targets. This finding is consistent with previous findings showing a several-fold increase in solubility for niclosamide cocrystals.[27] Pharmacologically, niclosamide has been shown to exert antiproliferative effects against various breast cancer cell lines, including MCF-7, MDA-MB-231, and T47D, with IC_{50} values in the micromolar range.[14,28]

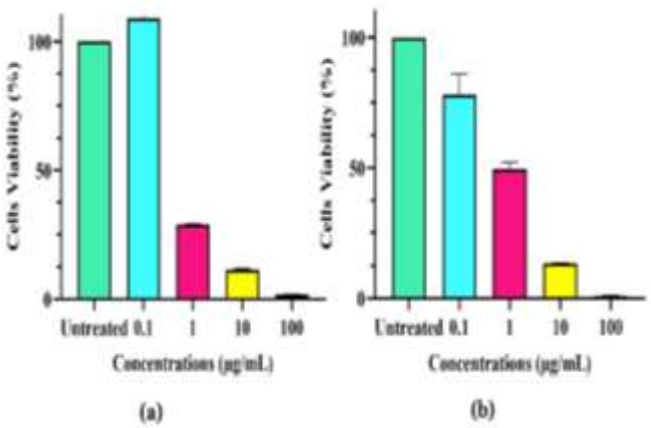

Figure 1: Cell viability (%) of T47D breast cancer cells after 48-hour treatment with **(a)** pure Niclosamide and **(b)** the Niclosamide–saccharin cocrystal on T47D cells. All values are expressed as means (n = 3) ± SD.

In the present study, the IC_{50} value of pure niclosamide in T47D cells (~3–4 µM when converted from µg/mL) is comparable to that reported by Li *et al*., supporting the validity of the current findings.[13] The lower IC_{50} observed for the cocrystal further confirms that physicochemical optimization contributes to enhanced cytotoxic effects without the need for chemical modification of the parent molecule. This aligns with the current trends in drug repurposing and solid form optimization, which are increasingly adopted to improve the therapeutic potential of existing drugs, particularly in oncology applications.[5,29,30]
From a mechanistic perspective, niclosamide is known to inhibit several critical signaling pathways in cancer cell survival, including STAT3, Wnt/*β*-catenin, NF-κB, and mTOR, all of which play key roles in proliferation, apoptosis resistance, and metastasis.[11,28,31,32] With improved solubility, the cocrystal form may enhance the rate and extent of active molecule penetration into cells, accelerating interactions with molecular targets and producing more substantial biological effects.[33,34] Therefore, these findings not only provide experimental evidence of enhanced cytotoxic potency through cocrystallization but also highlight the relevance of solid form modification strategies in the development of more effective anticancer drug formulations.

Microscopic observation showed that the untreated T47D breast cancer cells exhibited a typical epithelial-like morphology with intact cell membranes and tightly adherent growth patterns. In contrast, cells treated with pure niclosamide and niclosamide–saccharin cocrystal displayed marked, concentration-dependent morphological alterations, including cell shrinkage, rounding, membrane blebbing, and reduced confluence, with the most pronounced effects observed at 100 µg/mL. At intermediate concentrations (10 and 1 µg/mL), partial detachment and decreased cell density were evident, whereas low concentrations (0.1 µg/mL) produced only minimal changes compared to the control, as illustrated in Figure 2. These morphological features are characteristic of both apoptotic and cytotoxic responses, consistent with previous findings in cancer cells, where structural disruption and cell rounding are associated with loss of viability and induction of apoptosis.[35,36] The more substantial morphological damage observed in the cocrystal-treated group compared to pure niclosamide at equivalent concentrations supports the enhanced cytotoxic potency of the cocrystal formulation, likely due to improved cellular uptake and interaction with intracellular targets.

Conclusion

This study demonstrated that the niclosamide–saccharin multicomponent crystal exhibits a significant cytotoxic effect against T47D breast cancer cells. The multicomponent crystal showed enhanced anticancer activity compared to pure niclosamide, with IC_{50} values of 0.90 µg/mL and 1.39 µg/mL, respectively. Both compounds demonstrated cytotoxic activity categorized as highly active based on IC_{50} classification criteria. These findings suggest that the formation of a niclosamide–saccharin multicomponent crystal may enhance the anticancer potential of niclosamide and could serve as a promising candidate for further development as an anticancer agent.

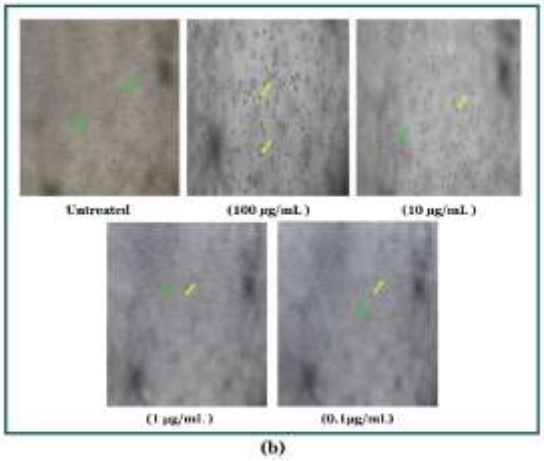

Figure 2: Morphological changes of T47D breast cancer cells after 48-hour treatment with **(a)** pure Niclosamide and **(b)** Niclosamide–saccharin cocrystal observed under an inverted microscope. The green arrow indicates viable cells, whereas the yellow arrow highlights cells exhibiting morphological alterations. Cell morphology was examined using an inverted microscope at 100x magnification.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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